IMMUNE RESPONSES IN A MOUSE MODEL OF *Lawsonia intracellularis* INFECTION.

Suzanne C. Mitchell

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Though knowledge must be got with pain,
And seemeth bitter at the root;
It brings, at last, a matchless gain,
And yieldeth forth most pleasant fruit.

George Wither. For Scholars and Pupils.

THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

(Regulation 3.5.13)

Name of Candidate ..... Suzanne Christina Mitchell .....

Address ..... Department of Veterinary Pathology, Easter Bush Veterinary Centre, Roslin .....

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Lawsonia intracellularis is a Gram negative obligate intracellular bacterium and the aetiological agent of proliferative enteropathy (PE, an enteric disease best characterised in pigs). The organism demonstrates tropism for the ileum and colon, multiplying freely in the cytoplasm of immature crypt enterocytes. In infected animals *L. intracellularis* can be demonstrated consistently intracellularly within the apical cytoplasm of immature crypt epithelial cells, which is associated with increased enterocyte proliferation and the loss of goblet cells. To date characterisation of immune cell infiltration has been rudimentary. Natural infection has been diagnosed in a variety of animals including hamsters, ferrets, horses, emu and deer, but has been most commonly detected in the pig, an animal in which it is prevalent world-wide. Experimentally challenged pigs and hamsters (using pure cultures of bacteria) reproduces the features of natural disease, but successful experimental infection in a mouse model has not been described. The aim of this thesis was to establish an infection system in the mouse and use this to examine the development of infection and lesions of proliferative enteropathy within the gastrointestinal tract. This mouse model was then used to assess the mucosal and serological responses generated by the host in response to inoculation with *L. intracellularis*.

The infection system demonstrated that 129/Sv/Ev wild-type and isogenic interferon gamma receptor null ($IFN\gamma R^{-/-}$) mice developed characteristic lesions of proliferative enteropathy following oral challenge with a pure culture of *L. intracellularis* (approximately $5x10^7$ organisms). These were directly comparable to the lesions seen in natural disease with increased proliferation of infected enterocytes in the ileum and colon associated with the loss of goblet cells.

Wild-type 129/Sv/Ev mice appeared capable of resolving or resisting infection with this obligate intracellular pathogen, with lesions of proliferative enteropathy detectable only at day 14 or 21 post inoculation, thereafter (days 28 and 35) there were none detectable. In comparison, $IFN\gamma R^{-/-}$ mice were highly susceptible to disease and appeared unable to eliminate infection over the time course studied, indicating that a functional IFNγ receptor is necessary in the control of infection.

Immunohistochemical analysis of immune cell markers including $CD3^+$, $CD4^+$, $CD8^+$, γδ TCR^+ and $CD103^+$ cells following inoculation with *L. intracellularis* was then assessed. Results indicated that there was a marked cellular infiltration following inoculation with *L. intracellularis*, in both WT and $IFN\gamma R^{-/-}$ mice. These changes included statistically significant differences (general linear model, $P<0.05$) between the $CD3^+$, $CD4^+$ and $CD8^+$ populations of inoculated and control WT groups over the 28 days assessed, as well as significant changes in the $CD4^+$ population between inoculated and control $IFN\gamma R^{-/-}$ mice. Notably, post-hoc analysis demonstrated some significant differences in cellular infiltration between infected WT and $IFN\gamma R^{-/-}$ mice at 14 days post-inoculation (the only time point where infection was demonstrable in both backgrounds).

This novel system is the first detailed description of proliferative enteropathy in mice and has demonstrated the usefulness of this model in the characterisation and analysis of important immunological events during infection with *Lawsonia intracellularis*.

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Declaration.

I declare that all of the investigations presented in this thesis, and its composition, are my own except where specifically stated.

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ABBREVIATIONS.

APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	DNA complementary to mRNA
DAB	3,3 diaminobenzedine
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
FCS	Foetal calf serum
H+E	Haematoxylin and eosin
HRP	Horse radish peroxidase
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFNγ	Interferon gamma
$IFN\gamma R^{-/-}$	Interferon gamma receptor knockout
IL	Interleukin
IPX	Immunoperoxidase buffer
kb	Kilobase
kDa	Kilodalton
KGF	Keratinocyte growth factor
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NMS	Normal mouse serum
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS with tween

PCR	Polymerase chain reaction
PE	Proliferative enteropathy
PHE	Proliferative haemorrhagic enteropathy
PIA	Porcine intestinal adenomatosis
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
SPG	Sucrose potassium glutamate buffer
TNF	Tumour necrosis factor
WT	Wild-type strain

1. INTRODUCTION.

1.1 *Lawsonia intracellularis.*

Lawsonia intracellularis, a Gram negative obligate intracellular bacterium, is the aetiological agent of proliferative enteropathy. It is a novel organism utilising intestinal enterocytes as a niche for survival and replication in an apparently unique manner. The key features of infection, and those which makes it interesting, are the increased level of proliferation seen in infected enterocytes, and the variable immune response documented to date. Infection with *L. intracellularis* results in the hyperplasia of immature enterocytes of the ileum and colon, a factor which exhibits species-wide distribution. As a prerequisite to detailed discussion of the development of proliferative enteropathy and associated immunological events in the mouse following inoculation with *L. intracellularis,* consideration will be given to some relevant key issues of the gastrointestinal immune system with particular emphasis on the role of epithelial cells.

1.2 Epithelial cell tropism.

L. intracellularis is an obligate intracellular bacterium and as such has an absolute requirement for multiplication within host cells. This dependence is also seen in *in vitro* cultivation, where the organism has not yet been maintained on any cell-free media (Lawson et al., 1993). Jacoby and Johnson (1981) initially demonstrated the persistence of the hamster *L. intracellularis* in cell culture. Stills (1991) was the first to maintain the hamster *L. intracellularis* in Int-407 cells, and also demonstrate that the cultivated bacteria were capable of producing disease.

Lawson et al. (1993) demonstrated the isolation and maintenance of the causative organism of porcine proliferative enteropathy in cell culture. This system enabled the bacteria to be maintained, over many passages, in the rat enterocyte cell line, IEC-18. Infected monolayers exhibited no detectable cytopathic effects during infection and passage of the bacteria (such as vacuolation, syncytia or inclusions), with the exception of heavily infected cells, which sometimes demonstrated a degree of vacuolation.

While IEC-18 cells were the preferred cell line for maintenance of *L. intracellularis,* growth could also be sustained in a variety of other cell lines (including human foetal intestine, Int-407; rat colonic carcinoma, CRL 1677; and pig kidney, PK-15), Lawson et al. (1993). Others, including pig intestinal lines proved less successful (IPEC-J2 while not enhancing growth did confirm the absence of any cytopathic affects in cell culture, McOrist et al., 1995b). The requirement of reduced oxygen tension for bacterial growth seen *in vivo* was also apparent with respect to *in vitro* cultivation, with optimal growth observed at a reduced oxygen tension of 8% O_2 (Lawson et al., 1993). Stills (1991) and Lawson et al. (1993) both used between 6-8% oxygen when detailing bacterial cultivation. However, Joens et al. (1997), succeeded in producing growth in an atmosphere modified only by the addition of 5% carbon dioxide. Some isolates will not grow in the presence of oxygen at atmospheric tension and may be killed by such exposure (Lawson et al., 1995); indicating that isolates may therefore vary in their susceptibility to oxygen (and possibly even indicating phenotypic differences).

Despite the fastidious nature of the organism there is still evident tropism for the epithelium and, more specifically, the rapidly dividing immature enterocytes within this environment.

1.3 Epithelial cell function and proliferation.

The initial function performed by the epithelial lining of the gastrointestinal tract is to act as a mechanical barrier against commensals, invading pathogens and food borne antigens, yet *L. intracellularis* manages to successfully colonise this protective boundary. This barrier must remain intact to act effectively, while also performing its other functions (for example absorption and digestion). The dynamics of maintaining an unimpaired and functional epithelium are themselves very complex, and will only be touched on here as an indication of the consequences of infection on disturbing this equilibrium.

The epithelium is in a constant state of renewal with epithelial cell division confined to the proliferative zones of the crypts of Lieberkuhn (Potten and Loeffler, 1990). Migration and differentiation of these cells then extends upwards, both in an anatomical and a functional sense. This involves the physical movement of the cells from the crypt onto the surface epithelium with associated lineage commitment and differentiation of these initially immature cells (Cheng and Leblond, 1974). The gastrointestinal stem cell has a distinct repertoire which includes all the main cell lineages observed in the gut, and is also responsible for the production of new intestinal crypts (Wong and Wright, 1999). Regulation and control of this process requires interactions between the epithelium and local heterotypic cells within the lamina propria (such as pericryptal myofibroblasts) and components of the extracellular matrix (such as laminin). The undifferentiated progenitor cells of the crypt provide a steady supply of replacement cells that migrate along the surface of the crypt and villus to become columnar absorptive epithelium and goblet cells that are eventually shed into the lumen from the exclusion zone at the tip of the villous (migration time is normally 2-3 days, Cheng and Leblond, 1974). Cell proliferation can be increased or decreased in response to physiological stimuli, for example altered food intake and pathological conditions. A complex array of interlocking factors are required to regulate the continued renewal of normal epithelium with the involvement of dietary influences and systemic hormones as well as the autocrine activities of transforming growth factor α (TGFα), epidermal growth factor (EGF) and transforming growth factor β (TGFβ) playing a major role.

The absence of goblet cells is a recognised feature of PE and suggests that infection with *L. intracellularis* may affect the differentiation of the immature epithelial cells. Furthermore infection with *L. intracellularis* has been clearly and consistently shown to be associated with the increased proliferation of crypt cells infected with the obligate intracellular pathogen. Whether the alteration in the cell cycle resulting in evident hyperplasia is due to the increased proliferation of the progenitor cells, a decrease in the number of cells maturing/differentiating or a decrease in cell apoptosis is not clear.

What can be demonstrated, however, is that intracellular infection with *L. intracellularis* has an as yet undetermined effect (whether directly or indirectly) on the immature crypt epithelial cells which it infects. Similar histological changes seen in other situations include evident crypt hyperplasia in biopsies of children with intolerance to cows' milk protein and gluten as well as following infection with *Giardia lamblia*, *Cryptosporidium* and enteropathogenic *E. coli* (Savidge et al., 1996).

In order to protect itself from pathogenic insult a susceptible host must be capable of monitoring, and responding, to any detrimental changes in its environment. Therefore epithelial cells, as one of the first barriers encountered following invasion by a micro-organism, are ideally suited to recognise an alteration in the luminal conditions. While originally thought only to act as a mechanical barrier, they are now known to both recognise invading pathogens and respond by signalling to immune effector cells. Kagnoff and Eckmann (1997) demonstrate that within the first few hours following bacterial (e.g. *Salmonella*) invasion, human colon epithelial cells are capable of producing mediators (e.g.IL-8, GRO-α, β, γ) that have the potential to orchestrate the onset of the mucosal inflammatory response. Due to the nature of the intestinal epithelium, and its constant interaction with luminal contents (including commensal organisms and their products) it must learn to act selectively, and avoid unnecessarily activating the hosts immune system. It is known that while pathogenic organisms are capable of generating an inflammatory immune response, commensal organisms commonly found within the gut lumen do not tend to activate this response. Svanborg et al. (1999) suggest that pathogenic bacteria have both a broader repertoire of host-activating molecules, as well as more efficient presentation of these molecules to the host cell. *L. intracellularis* is considered not to induce an inflammatory response therefore different factors may be involved.

1.3.1 Altered enterocyte proliferation and dysregulation by bacteria.

A characteristic feature of the lesions induced by *L. intracellularis* is the prominent hyperplasia of infected enterocytes, with a dramatic contrast seen between adjacent areas of healthy and diseased gut. The extent of epithelial proliferation, however, varies among the different species in which *L. intracellularis* causes proliferative enteropathy. For example while the fox (Ericksen and Landsverk, 1985), horse (Duhamel and Wheeldon, 1982) and guinea pig (Elwell et al., 1981) show hyperplasia of enterocytes; in hamsters (Frisk and Wagner, 1977; Jacoby, 1978) and pigs this can develop into adenomatous lesions; and in the rat (Vandenberghe et al., 1985) and ferret (Fox et al., 1989) an apparently carcinomatous condition develops, with metastases to local lymph nodes (although the presence of *L. intracellularis* was not confirmed in the rat by Vandenberghe et al., 1985). Crypt distortion and branching are commonly associated with inflammatory bowel disease and such affects have also been observed following infection with both *Shigella* and *Salmonella* (Sachdev et al., 1993), and this altered morphology may be as a result of cellular infiltration, or chronicity of disease. Islam et al. (1994) detailed a similar scenario of hyperplastic and branching crypts (as seen in PE) in *Shigella* infection in the colon (also in association with a reduction in the number of goblet cells). Proliferation of crypt epithelial cells was seen at sites distant from the primary ulcers, indicating a role for factors released from *Shigella* organisms in the induction of the hyperplastic state e.g. through a direct effect on the cells, or induction of growth factors such as epidermal growth factor (EGF). EGF secreted by gastrointestinal stem cells is thought to stimulate cell proliferation, regeneration and healing of chronic ulcers in patients with Crohn's disease and peptic ulcer disease, and so may play a role in the healing process (Wright et al., 1990). The presence of a mitogen has also been suggested as the cause of increased cell proliferation following infection with *Bartonella bacilliformis,* a Gram negative intracellular bacterium known to cause endothelial cell proliferation in the dermal verruga of Bartonellosis (Garcia et al., 1990, and Arias-Stella et al., 1986). It is indeterminate, however, if the presence of a secreted factor is the cause of proliferation seen in PE, since hyperplasia is exclusively seen in crypts infected with *L. intracellularis*, and does not appear at sites distal to infection.

Cell proliferation induced by *Citrobacter rodentium*, the causative organism of transmissible murine colonic hyperplasia is immune mediated, with disease resulting in crypt hyperplasia, epithelial cell proliferation and mucosal thickening (Higgins et al., 1999a). Proliferation was said to be due to immunopathology following a Th1 response, driven by the organism (and dependent upon the adhesin intimin), Higgins et al. (1999b). Epithelial proliferation induced by *Helicobacter pylori* is another example of bacterial driven immunopathology (Zarrilli et al., 1999). Infection with *H. pylori* triggers the release of EGF-related growth factors thereby stimulating cell proliferation. The organism also inhibits apoptosis and sustains the inflammatory response to promote disruption of mucosal integrity.

Cells involved in the maintenance of the mucosal equilibrium include myofibroblasts of the lamina propria. These cells are responsible for the production of matrix metalloproteinases (MMPs) which slowly degrade the matrix, tissue inhibitors of MMPs to prevent excess enzyme activity, and keratinocyte growth factor (KGF). As such they maintain the shape of the mucosa, and its integrity. In response to inflammation and associated elevated cytokine levels myofibroblasts increase production of these factors resulting in net mucosal growth and tissue remodelling (MacDonald et al., 1999) following disruption of the mucosal equilibrium. A further feature of tissue growth/remodelling in the intestine is associated villous atrophy and crypt hypertrophy, a feature of porcine PE (Lawson and Gebhart, 2000).

KGF production (by myofibroblasts) is upregulated following activation of T cells by superantigens and cytokines such as TNFα (Bajaj-Elliot et al., 1998). KGF then activates epithelial cells to make TGFα (which acts in an autocrine manner to increase crypt cell division) as presumably occurs during infection with *C. rodentium* (Higgins et al., 1999a). TGFα and TGFβ affect epithelial cell renewal both in a paracrine and autocrine fashion. Transforming growth factor β is an autocrine hormone normally found in villous cells that inhibits enterocyte proliferation (Barnard et al., 1989 and Moses et al., 1990).

Expression is most abundant in terminally differentiated villous tip cells and less prevalent in the less differentiated, mitotically active crypt cells (Barnard et al., 1989) suggesting that $TGF\beta_1$ may function to arrest growth as the enterocyte leaves the proliferative zone and to maintain the terminally differentiated state, but a direct role in enterocyte differentiation was not shown.

McOrist et al., 1992, suggested that the reduced number of villous cells in the terminal ileum of pigs with lesions of proliferative enteropathy could reduce local TGFβ expression and therefore reduce the inhibition of crypt cell proliferation, adding to the lesion's progression. Alternatively the proliferation of crypt cells (and their altered cell cycle and prevented maturation) may be the cause of the reduced number of villous cells and subsequent destruction of villous epithelium.

While the factors resulting in enterocyte hyperplasia following infection with *L. intracellularis* are unknown, it is clear that analysis of both bacterial and host driven aspects would be necessary to establish the pathogenic mechanisms involved in lesion development.

1.3.1.1 Apoptosis.

Apoptosis is a normal part of the dynamic epithelial cell cycle responsible for the constant regeneration of the mucosa. It can also be induced following bacterial invasion as a means of protecting the host from the spread of infection. Apoptosis is linked to the activation of the early epithelial cell inflammatory programme (Kagnoff and Echmann, 1997). Kim et al. (1998) demonstrate that, as in the case of cytokine production, apoptosis is a conserved response of intestinal epithelial cells to bacterial invasion. Bacteria using different strategies for host cell invasion and uptake, and with different intracellular locations e.g *Salmonella* within membrane vesicles and enteroinvasive *Escherichia coli* which escapes into the cytosol both induce apoptosis in a variety of different human intestinal epithelial cell lines. *In vivo* this results in the deletion of epithelial cells (with little inflammatory response), due to the phagocytosis or shedding of the cells.

Group A *Streptococcus* is also capable of inducing the apoptosis of cultured epithelial cells, with internalisation of the organism a prerequisite for commitment to the pathway (Tsai et al., 1999). Following bacterial invasion of the cell, TNFα and nitric oxide are produced. These factors are known to be important in the intestinal proinflammatory response, as well as regulating the apoptotic death of the cell. Apoptosis functions to remove infected and damaged cells, and to restore the integrity and normal growth regulation of the mucosal barrier. As a result of apoptosis, and increased cell growth to compensate for this loss, mucosal infection can result in increased crypt mitotic activity (Mathan and Mathan, 1991 and Savidge et al., 1996), crypt hyperplasia and branching, and increased epithelial cell proliferation with heaping up of epithelial cells (Islam et al., 1994 and Sachdev et al., 1993). Whether apoptosis has a role in the clearance of *L. intracellularis* infection is unknown, but the presence of an increased number of apoptotic cells is not a documented feature of disease pathology. The exact contribution of alterations in the rate of apoptosis and mitosis to the lesions of PE have yet to be elucidated. Increasing availability of reagents against cellular markers of apoptosis and proliferation should facilitate studies of this nature.

1.4 Epithelial cells as immune effector cells

Epithelial cells and T cells of the gastrointestinal tract demonstrate a degree of co-dependency with both cells relying on the other for signals that aid in their functional control including cell proliferation and differentiation. The preceding section dealt with the normal growth and control of epithelial cells, and the role played by growth factors. The following section aims to introduce the role of epithelial cells as functional immune effector cells with an important role in the development and maintenance of an effective intestinal immune response.

1.4.1 Epithelial cells as antigen presenting cells

For the generation of an effective specific immune response there must be suitable presentation of the intracellular bacterial antigens to the circulating cells of the immune system and this is the role of professional antigen presenting cells. Antigen presentation occurs primarily in the gut-associated lymphoid tissue (GALT) where it induces the activation and proliferation of naïve intestinal T lymphocytes. Adequate activation of the T cells requires an array of co-stimulatory factors such as MHC antigen presentation and engagement of the TCR-CD3 complex, B7, and IL-2. *L. intracellularis* preferentially invades enterocytes, and these are classed as non-professional antigen-presenting cells (and are unique as such in their constitutive expression of MHC II in the small bowel and colon (Mason et al., 1981; Scott et al., 1980; Wiman et al., 1978)). MHC II is seen on normal intestinal epithelial cells in the mouse, rat and human (Bland, 1988). The principal differences between these cells and professional antigen presenting cells (APCs) of haematopoietic origin (such as dendritic cells, macrophages and B cells) include the method used to transcytose antigen and the lack of co-stimulatory molecules (e.g. B7-1 and B7-2); as well as the expression of non-polymorphic restriction elements, such as CD1d. The absence of such molecules led to the suggestion that epithelial cells may induce anergy, rather than activation, of naïve T cells (Bland and Warren, 1986). Epithelial cells have also been shown to mediate antigen non-specific suppression of both T- and B-cell mediated immune responses. Therefore while the potential role for epithelial cells to be directly involved in antigen presentation has been demonstrated *in vitro* (Mayer, 1997) it requires more detailed investigation.

1.4.2 Epithelial cell cytokine production.

Kagnoff and Eckmann (1997) termed the conserved early and late responses of epithelial cells to invasive pathogens, the 'epithelial cell inflammatory gene programme'. This 'programme' includes aspects of the immune response such as the regulated expression and production of proinflammatory cytokines, prostaglandins, nitric oxide and increased ICAM-1 expression.

The varied kinetics of production, alongside quantitative differences in chemokines secreted by epithelial cells, suggest that these cells play a more complex role in immune modulation than originally thought. High levels of C-X-C chemokines (including IL-8, and characterised by their ability to chemoattract and activate polymorphs) were released following epithelial cell stimulation and, at a lower level, C-C chemokines such as MCP-1, MIP-1β, MIP-1α and RANTES (factors which can act as chemoattractants of monocytes/macrophages, eosinophils, and subpopulations of T cells). This implies that epithelial cells are capable of generating a varied response resulting in the induction of a number of different inflammatory cells. Other proinflammatory cytokines secreted include TNFα, GM-CSF, IL-1α and IL-1β (although at lower levels than that seen for the chemokines). The upregulated expression of immunoregulatory cytokines by epithelial cells in response to enteroinvasive pathogens is qualitatively similar following varied insults of micro-organisms, therefore suggesting the evolution of conserved functions in response to a broad array of different invasive bacteria, irrespective of particular bacterial invasion strategies (e.g. different methods of entry and choice of replication site utilised by intracellular pathogens such as *Yersinia, Salmonella, Shigella* and *Listeria* elicit a similar response). Upregulated expression of proinflammatory cytokines is seen quickly (within 2-3 hours) after bacterial invasion, but the decline is also rapid. This suggests that the cells may be important for signalling the onset of infection; but that following the recruitment of inflammatory cells, the level of epithelial cell cytokine production is reduced, and taken over mainly by monocytes/macrophages and other inflammatory cells. This is not the case for all released chemokines, with both ENA-78 and RANTES (neutrophil and T cell chemoattractants) expressed following a delayed response by the intestinal epithelial cells (as are the cytokines linked with apoptosis).

Despite the aforementioned release of proinflammatory cytokines, there is no evidence for the production of IL-2, IL-4, IL-5, IL-12 p40, or IFNγ (Jung et al., 1995) by intestinal epithelial cells.

These latter cytokines are generally linked to the generation of an antigen–specific immune response, suggesting epithelial cells may play an important role in the generation of innate immunity rather than a specific immune response.

However, in contrast to this apparent inability to produce cytokines associated with the specific arm of the immune response they may still be involved via the expression of membrane proteins capable of responding to these cytokines. This is supported by the expression of cellular receptors for a variety of cytokines including IFNγ, IL-1, TNFα, TGFβ1, as well as IL-2, IL-4, IL-7 and IL-9 (Reinecker and Podolsky, 1995).

The epithelium is therefore important in activation of the immune response especially via attraction of polymorphonuclear cells and macrophages. These antigen presenting cells then process and present antigen to activate specific immune defences via T- and B- lymphocytes.

1.5 T cells in the gastrointestinal tract.

The gastrointestinal tract has a large number of associated T lymphocytes and, as well as being distinct from other T cells within the body, they are especially well adapted to the particular requirements of the intestine. For example recently Huleatt et al. (2001) demonstrated that following infection with *Listeria monocytogenes* the $CD8^+$ cell populations vary between the spleen and the intestinal mucosa. While the lymphocytes overlap with regards to antigen specificity, they are distinct in terms of their repertoire, which ultimately reflects their function *in situ.* For example the frequency of MHC class I-a or I-b restriction, and the variation in TCR Vβ repertoires between the two sites. This supports the idea that intestinal T lymphocytes can be regarded as a highly complex and unique area within the immune system.

Intestinal T cells occur principally in 3 major compartments: (a) organised gut-associated lymphoid tissue (GALT) such as Peyer's patches; (b) the mucosal lamina propria; and (c) the surface epithelium. Even within these compartments the phenotypic distribution varies therefore illustrating that there are further levels of specialisation.

Current concepts in gastrointestinal immunology indicate that antigen-induced activation, proliferation and partial differentiation of naïve intestinal T lymphocytes occurs primarily in GALT. It is assumed that these primed cells then migrate to mesenteric lymph nodes, where they further differentiate, and then follow the thoracic duct lymph into the peripheral blood circulation. Consequently the memory/effector cells progress to mucosal tissues by relatively site-specific homing mechanisms. The adhesion molecules expressed by different T cell phenotypes, as well as those on endothelial cells, direct the specificity of an immune response, resulting in cells of a particular phenotype being directed towards specific sites (Brandtzaeg et al., 1998). Naïve and memory lymphocytes flow through high endothelial venules where they interact with the endothelium through a weak, rolling interaction. This initial binding is, in many cases, mediated by the interaction of selectins with glycosylated ligands. For some adhesive interactions, the rolling interaction is enhanced by the presence of chemokines. As the lymphocyte rolls along the endothelium, chemokine receptors expressed on the T cell clone come into further contact with chemokines displayed within the endothelial cell glycocalyx. If the chemokine receptor encounters an appropriate chemokine, it triggers integrins to bind their immunoglobulin superfamily member ligands with greater avidity. As a result of this interaction, the lymphocyte binds more firmly, stops rolling, and then extravasates through the endothelium and into the tissue. Lymphocytes are maintained within the tissue by additional chemokine signals and adhesive interactions, which might include the binding of integrins to extracellular matrix or cellular counter-receptors expressed on cells within the tissue.

1.5.1 Adhesion molecules

A variety of homing receptors and chemotactic factors direct T cells extravasation to the appropriate area of inflamed intestine. Thereafter the T lymphocytes can be categorised further based on their location within the gut mucosa, with some residing within the lamina propria, and others becoming intraepithelial lymphocytes. Both intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) are believed to be previously activated/memory cells, recruited to the intestinal mucosa following activation in gut-associated lymphoid tissues. This process of lymphocyte recruitment involves a variety of secreted and membrane-bound factors, including adhesion molecules involved in rolling interactions.

Adhesion molecules expressed by epithelial cells include ICAM-1, LFA-1, E-cadherin and CD23. These molecules have a role in the interaction of host cells with T cells in the immune response against infection. For example ICAM-1 is constitutively expressed on human gut epithelial cell lines (HT29 and Caco-2), is upregulated by IFNγ and IL-1β and mediates adhesion to activated T lymphocytes through ICAM-1/LFA-1 interactions (Kaiserlian, 1999). Although ICAM-1 is not found on mature epithelial cells, it is induced on crypt epithelial cells and on the rectal epithelium during graft-versus-host disease (GVHD) concurrent with the presence of surrounding LFA-1-positive intraepithelial cells (Norton et al., 1992).

Expression of ICAM-1 on (human) enterocytes *in vivo* is upregulated following infection with invasive bacteria (enteroinvasive *E. coli, Y. enterocolitica, S. dublin, L. monocytogenes*) and this coincides with increased neutrophil adhesion to these cells, Huang et al. (1996). Therefore indicating that ICAM-1 may maintain neutrophils that have transmigrated through the epithelium in close contact with the intestinal epithelium, and therefore reduce further invasion of the mucosa by invading pathogens. Shibahara et al. (2000) have also shown ICAM-1 expression on lymphocytes and its significant up-regulation during IEL retention in the epithelia (this was not mediated by epithelial derived factors).

$\alpha_4\beta_7$ is a lymphocyte adhesion molecule that directs lymphocyte homing to the intestine. The integrin is a receptor for mucosal addressin cell adhesion molecule-1 (MadCam-1), a glycoprotein expressed on intestinal lamina propria venules and on high endothelial venules in Peyer's patches and mesenteric lymph nodes (Berlin et al., 1993).

E-cadherin is constitutively expressed on normal intestinal epithelial cells, and mediates the interaction between the intestinal cells and intraepithelial lymphocytes (IEL) via the $\alpha_E\beta_7$ (CD103) ligand (Cepek et al., 1994) thereby allowing IEL cytotoxicity to be directed towards the epithelial cells.

CD23 is the low affinity receptor for the Fc portion of IgE, as well as a receptor for the adhesion molecule CD21. It is constitutively expressed on normal human enterocytes, and is enhanced during an inflammatory response (Kaiserlian, 1999).

A number of epithelial ligands therefore may be important in directing lymphocytes to the intestinal mucosa. Due to the potentially important role for E-cadherin in the recruitment and maintenance of intraepithelial lymphocytes at sites of infection, as well as its function in maintaining cell structure, this molecule will be looked at in more detail. E-cadherin belongs to a family of homophilic cell adhesion proteins and forms adherens junctions on the lateral surfaces of epithelial cells. It plays a critical role in cell sorting during development, formation of intercellular junctions and maintenance of tissue architecture. The extracellular region consists of five tandemly repeated domains that require calcium binding for their function and stability (Yoshida-Noro et al., 1984; Grunwald, 1993). In epithelial tissues the adherens junctions form a belt around each cell, creating a continuous zipper of cell adhesion (Shapiro et al., 1995). On the cytoplasmic side of the cell membrane E-cadherin interacts with either β- or γ-catenin, and α-catenin then links this to the actin cytoskeleton. The catenin binding domain of E-cadherin is required for cell adhesion, but is thought to play a regulatory, rather than a mechanical, role (Nagafuchi et al., 1994).

β-catenin is a key regulator of both cell proliferation and migration and has also been identified as a second messenger in the signal transduction cascade of Wnt proteins (which are involved in control of tissue polarity, Blankestejin et al., 2000). The degradation of β-catenin is regulated by the adenomatous polyposis coli protein, and elevated levels of β-catenin are more likely to occur indirectly as a result of mutations in this protein than as a direct consequence of mutations in the β-catenin protein (Kitaeva et al., 1997). The adenomatous polyposis coli protein is thought to modulate the interaction between cadherins and catenins, thereby affecting the pathway through which intercellular interactions control cell growth and differentiation (Su et al., 1993). In fact the loss of the adenomatous polyposis coli protein appears to be an extremely early event that initiates colon cancer (Powell et al., 1992).

Thus in addition to lymphocyte homing and activation some of these ligands may have importance in regulation of cellular activities such as proliferation.

1.5.2 Lamina propria lymphocytes.

Lamina propria lymphocytes (LPL) consist primarily of T cells (40-90%) and the majority of these employ αβ TCR. Furthermore within this group 65-80% of $CD3^+$ cells are also $CD4^+$ (Beagley et al, 1992). A much smaller proportion of LPL are B cells, with the majority of these being plasma cells (Beagley et al., 1992). Those LPL that are $CD4^+\alpha\beta$ TCR tend to all express the integrin $\alpha_4\beta_7$ with a proportion also expressing $\alpha_E\beta_7$. Many possess features indicating an active phenotype, for example they are L-$selectin^{lo}$, $CD69^+$ (putative marker of recent activation), $CD45RO^+$ (memory marker), $CD25^+$ (marker of recent activation), and Fas^+ (Schieferdecker et al., 1992; De Ruggero et al., 1992).

As with all areas of the immune system there must be a degree of control exerted over all cell activity, including cell death, to prevent an inappropriate immune response. To this end the activated T lymphocytes found in the lamina propria are programmed to die unless rescued by antigen or chemokines, and the expression of Fas and FasL on these T cells indicates that the majority die *in situ*.

In contrast to these 'death factors' interactions between epithelial produced IL-7 and lamina propria T cells are a means of positively regulating cell proliferation (Watanabe et al., 1995). Within this group of LPL γδ cells are uncommon (Farstad et al., 1996), and it is not clear if $CD8^+$ cells found in the lamina propria are resident or in transit to the epithelium.

Regulation of the immune response is also required, as is generation of the effective Th1 or Th2 type response depending upon the specific insult. IL-12 drives the formation of a Th1 response, whereas IL-4 is responsible for the generation of a Th2 response. There is also positive feedback amplification of IL-12 production mediated by IFNγ and this represents a potentially disadvantageous mechanism leading to uncontrolled cytokine production. The immune system is then capable of employing its own method of down-regulation and using this for its own advantage and protection when the infection has been successfully eliminated. IL-12 is capable of inducing both IFNγ to promote, and IL-10 (Finkelman et al., 1994) to suppress its own production.

The cytokine interleukin 12 acts as a bridge between the early non-specific innate resistance, and the subsequent antigen-specific adaptive immunity. The heterodimer is produced by a variety of cells (including phagocytes and B cells) in response to infection, and then effects a variety of responses in, principally, NK and T cells. IL-12 induces cytokine production (primarily of IFNγ) from NK and T cells (Manetti et al., 1993), acts as a growth factor for activated NK and T cells, and enhances the cytotoxic activity of NK cells and favours CTL generation (Gately et al., 1992).

Cell production of IL-12 is governed by those cells capable of expressing the p40 gene, since the molecule is only effective as a heterodimer of 70kDa consisting of two covalently linked chains of approximately 40 (p40) and 35 (p35) kDa. Transcripts of the p35 genes are constitutively expressed, albeit it a low level, in most cell types analysed. As mentioned previously IL-12 p40 has not been detected in epithelial cells.

IL-12 induction of IFNγ requires the presence of accessory cells, in contrast to the stimulatory effect that IL-12 has on NK cytotoxicity which does not.

IFNγ can inhibit the proliferation of Th2 cells, but not Th1 cells, due to the absence of the second chain of the IFNγR (IFNγRβ or accessory factor 1) on the Th1 cells (Pernis et al., 1995). This down-regulation allows the immune system to selectively inhibit the proliferation of Th2 cells, without affecting that of the IFNγ producing Th1 cells. IFNγ, in its role as a Th1 cytokine, also demonstrates the ability to down-regulate MHC expression and antigen processing in B cells (O'Neil et al., 1999) and therefore inhibit the generation of a humoral immune response. This is in direct contrast with its function on macrophages (as well as a variety of other cells) to up-regulate MHC expression, leading to improved antigen presentation to $CD4^+$ and $CD8^+$ T cells. In monocytes/macrophages IFNγ is also capable of altering other functions such as the rates of endocytosis/phagocytosis, and the levels of lysosomal proteinases to promote protection against invading pathogens, and may be important in infection with *L. intracellularis*.

1.5.3 Intraepithelial lymphocytes.

Whereas LPLs are predominantly $CD4^+$ (see Table 1.1), and also include a substantial population of B cells in addition to T cells, IELs by comparison are predominantly $CD8^+$. IEL from adult mice are 40-70% αβ TCR and 30-60% γδ TCR compared to only 10-15% γδ TCR expression in man (Jarry et al., 1990 and Vankerckhove et al., 1992).

Intraepithelial lymphocytes (IEL) are largely extrathymically derived (and may mature within the intestinal environment), have a limited TCR repertoire capable of recognising common microbiologic antigens, and demonstrate predominantly cytolytic functions. In contrast LPL are thymically derived and highly activated lymphocytes. The proximity of IEL to bowel lumen means that they are the first component of the mucosal immune system to come into contact with bacterial and food antigens. Not only do IELs differ in both phenotype and function from LPL, but there is also variation among regions of the gastrointestinal tract.

For example there are approximately 20-30 times more small intestinal IELs than colonic IELs (Beagley et al, 1995). Colonic IEL express higher levels of mRNA for IL-2, IL-4, IL-5, and IL-10 but similar amounts of IL-1, IFNγ, and TNFα when compared to small intestinal IEL (Beagley et al., 1995). High expression of CD69 by colonic IELs suggests that these lymphocytes are also chronically activated (Ibraghimov and Lynch, 1994). A clear variation can also be seen among the distribution of $CD4^+$ and $CD8^+$ IELs between different strains of mice (Beagley et al., 1995). This further indicates the high degree of specialisation within the mucosal T lymphocyte lineage.

Cell surface markers	IEL	LPL	PBL
CD4+	20%	65%	65%
CD8+	80%	35%	35%
αβ TCR	50%	95%	95%
γδ TCR	50%	3%	3%
HML-1 ($\alpha^E\beta^7$+)	90%	30-40%	0%
CD25 (α-chain IL-2R)	15-30%	15-30%	<5%
CD45RO+	85%	65-95%	30-50%
CD45RA+	15-20%	10-22%	60-90%

Table 1.1. Phenotypic markers of intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) compared with peripheral blood lymphocytes (PBL). From Abreu-Martin and Targan (1996).

Contributory roles for intestinal lymphocyte localisation, of both lamina propria and intraepithelial lymphocytes, may be exercised by both the expression of integrins, such as $\alpha_4\beta_7$, $\alpha_E\beta_7$ and $\alpha_1\beta_1$ for homing and IEL retention (Roberts et al., 1999), along with the selective expression of chemokines and their receptors. Agace et al. (2000) have suggested a potential role for CCR2, CXCR3 and CCR5 in lymphocyte localisation within the intestinal mucosa (with constitutive expression of their ligands such as MCP-1, IP-10, MIP-1α, MIP-1β and RANTES being upregulated in the inflamed intestine (Grimm and Doe, 1996)).

CCR9 and CXCR4 are also regarded as potential candidates for mediating lymphocyte localisation and trafficking (Zabel et al., 1999).

Cytokines released by IELs, such as IFNγ or KGF modulate surface expression of adhesion molecules on epithelia and may modulate epithelial cell growth (Shibahara et al., 2000, Boismenu and Havran, 1994, and Colgan et al., 1996). Conversely epithelial cells are also capable of exerting regulatory forces on lymphocytes such as the secretion of chemotactic factors (Warhurst et al., 1998), or those capable of regulating the proliferation and immune functions of IELs (Yamamoto et al., 1998. Christ et al., 1997, Watanabe et al., 1995). *In vitro* studies have also demonstrated that epithelial cells can present antigen to T cells (Mayer and Shlien, 1987 and Mayer et al., 1991) therefore further indicating the potential role for epithelial cells in a developing immune response.

A principal *in vitro* (and presumably *in vivo*) function of IELs is their constitutive cytolytic activity (Goodman and Lefrancois, 1988). Both antigen-specific and non-specific CTL function has been described in murine $\alpha\beta^+$ TCR and $\gamma\delta^+$TCR IEL. This is thought to be an important rapid response mechanism in host defence against invasion by pathogens and in destruction of transformed epithelial cells. γδ T cells can recognise evolutionary conserved sequences such as heat shock proteins (Kluin et al., 1991), and rotavirus-infected epithelial cells (Offit and Dudzik, 1989).

γδ IEL, but not αβ IEL, have genetically determined cytolytic activity and IFNγ-producing ability (Ishikawa et al., 1993). They further suggested that within the IEL compartment, the more evolved αβ T cells depend on antigenic stimulation for differentiation and function, whereas the more 'rudimentary' γδ T cells develop in the absence of antigenic stimulation and have cytolytic potential that is genetically predetermined. Because of the pre-determined cytolytic activity of γδ TCR^+ cells they may be particularly well placed to recognise and eliminate transformed cells as a result of intracellular bacterial infection, such as *L. intracellularis.*

A potential role for the helper activity of IELs also exists as a result of their cytokine production. All IELs have the ability to secrete cytokines associated with both Th1 and Th2 function, therefore suggesting that IELs may be capable of regulating other lymphocyte populations as well as epithelial cell function such as expression of MHC I and secretory component (Brandtzaeg et al, 1988).

1.5.4 Gamma delta TCR$^+$ lymphocytes.

While the majority of T lymphocytes within the gastrointestinal tract are αβ TCR$^+$, there are a significant subdivision grouped by their γδ TCR. These comparatively uncharacterised cells possess a variety of unique properties which include their expression of the conserved γδ TCR. This extends to their distinct pathway of development (and subsequent anatomical distribution), their antigen specificities (and intercellular interactions), and to a non-redundant role in immune responses against infection. It is hypothesised that γδ T lymphocytes do not routinely rely on professional APCs for antigen recognition, and this is supported by the relative absence of γδ T cells from regions such as the lymph nodes and spleen where antigen is customarily presented to the residing population of naïve lymphocytes. Instead the cells have a tendency to localise in tissues, such as the intestine, skin or lung (Itohara et al., 1990) where they may recognise antigens directly, sometimes using limited TCR γδ diversity (Janeway et al., 1988). This lead to a 'first line of defence' hypothesis (Janeway et al., 1988) proposing that γδ cells responded to an imbalance in the host (such as inflammation, cell transformation or tissue damage, perhaps involving the expression of unique 'stress antigens') rather than a diversity of microbial antigens.

Peripheral γδ T cells are predominantly DN (CD4$^-$/CD8$^-$) whereas γδ IELs have a high expression of the CD8αα+ homodimer (Lefrancois, 1991). The absence of conventional CD4 and CD8 expression further indicates the altered function of γδ T cells, for example non-conformity to the classic MHC recognition by CD4$^+$ or CD8$^+$ αβ TCR$^+$ lymphocytes (Schild et al., 1994). The context in which the γδ T cell does recognise antigen, however, is not yet clearly elucidated.

In many ways the functions of γδ TCR^+ are easily substituted for (and therefore hidden) by αβ TCR^+ cells, in part due to some shared characteristics where a prevalent γδ cell function is unclear. For example both populations possess cytolytic effectors, perforins, granulysin, fas/fasL, and produce IFNγ. Similarities are also apparent in the differential production of cytokines such as IL-2 and IFNγ, and IL-4, IL-5 and IL-10 that distinguishes Th1 and Th2 αβ T cells respectively (Ferrick et al., 1995), with γδ cells expressing a predominantly Th1 phenotype. On activation intestinal $\gamma\delta^+$ intraepithelial lymphocytes secrete IL-2 and become cytolytic, as well as up-regulating lymphotactic chemokines such as MIP1α, MIP1β, RANTES and lymphotactin which can recruit $CD8^+$ T cells (Boismenu et al., 1996). In this respect it is apparent that γδ T cells might respond to stimuli expressed by epithelial cells and may then be capable of exerting effector functions either directly or indirectly on these target epithelial cells. γδ cells may also have a role in the maintenance of epithelial integrity via the production of growth factors such as the keratinocyte growth factor (KGF, Boismenu and Havran, 1994) which induces epithelial cell proliferation and is involved in epithelial repair. Peripheral γδ cells can be strongly activated by a combination of IL-1 and Il-7, and are significantly more vulnerable than αβ T cells or NK cells to IL-7 deficiency. This is interesting given that epithelial tissues can be a source of IL-7 (Watanabe et al., 1995).

1.6. Humoral immunity.

There is a significant functional role for B cells in the intestine as well as the secretion of mucosal and serological antibodies. They function as professional antigen presenting cells and helper cells in the generation of a specific immune response. The dominant immunoglobulin secreted by B lymphocytes in the gastrointestinal tract is IgA, accounting for 70-90% of all immunoglobulin present in normal intestinal mucosa. Secretory IgA is produced by B cells in the mucosal lymphoid follicles and is taken up by the basolateral surface of epithelial cells and released into the bowel lumen where it prevents invasion of epithelial cells by pathogens.

B cells are first exposed to environmental antigens in the B cell zone of the PP (McGhee et al., 1989).

The immune system is involved in a highly complex interaction with the mucosa and is intimately involved not only in the defence against pathogens, but also in epithelial proliferation. There are several infectious diseases in which changes to epithelial proliferation are evident, including proliferative enteropathy in which many facets of host responses remain relatively uncharacterised.

1.7.Proliferative Enteropathy.

Proliferative enteropathy (PE) is an enteric disease with an undefined host range, recognised as particularly affecting pigs (McOrist et al., 1993). It is found multiplying free in the cytoplasm of immature enterocytes principally within the ileum and colon. Originally given the vernacular name of *Ileal symbiont intracellularis* (Gebhart et al., 1993), it was later designated genus *Lawsonia*, species *intracellularis* (McOrist et al., 1995a) and is the sole member of the genus.

PE was first described in 1931 as a case report (Biester and Schwarte, 1931) with the presence of bacteria within lesions first documented in 1973 (Rowland et al., 1973). PE was initially thought to be caused by a campylobacter due to the various organisms of that genus (mainly *Campylobacter mucosalis* and *Campylobacter hyointestinalis,* Love et al., 1977; Ohya et al., 1985; Ericksen et al., 1990; Alderton et al., 1992) that were isolated from typical lesions. However, while disease could be reproduced by exposing animals to diseased mucosa (Jacoby et al., 1975; Roberts et al., 1977; Mapother et al., 1987), no disease or intracellular colonisation occurred following transmission studies with *Campylobacter* spp. (McCartney et al., 1984). Additional studies showed that monoclonal and polyclonal antibodies directed against the intracellular bacteria consistently present within lesions did not recognise known *Campylobacter* spp. (Lawson et al., 1985). Further to this, rabbit antisera prepared against whole cells of *Campylobacter* sp. did not react with the intracellular organisms in indirect immunofluoresence assays.

Supplementary support indicating that the causative organism was not a *Campylobacter* came when antisera prepared against the intracellular organism, purified from lesions by selective filtration, was shown to react with intracellular bacteria in sections of lesions from other pigs (Lawson et al., 1985) but not *Campylobacter*.

Both monoclonal (McOrist et al., 1987) and polyclonal (Lawson et al., 1985) antibodies raised against this intracellular agent therefore distinguished it from any campylobacter organism previously thought to be associated with disease aetiology. The unique nature of the causative organism of PE was further established by the generation of cloned DNA probes that discriminated between the intracellular organism and all known porcine *Campylobacter* species (Gebhart et al., 1991). This led to specific *in situ* hybridisation studies which demonstrated correlation between the sites of localisation of the intracellular bacteria (seen originally by silver-staining) with hybridisation of the specific DNA probe for the organism (Gebhart et al., 1991). Additional studies noted that the outer membrane protein profile and the fragment pattern of DNA restriction endonuclease digests were inconsistent between the intracellular bacteria and *Campylobacter* sp. (McOrist et al., 1990). These results therefore conclusively separated this novel intracellular bacterium from *Campylobacter* species.

Gebhart et al., 1993, proposed the interim name *Ileal symbiont intracellularis* for the organism. A taxonomic study in which the 16S ribosomal DNA (rDNA) of isolates from four pigs were amplified and sequenced revealed that the purified intracellular organisms were homogeneous, and distinct from any known bacteria. They further demonstrated that the organisms belonged to the delta subdivision of the class *Proteobacteria,* and also illustrated a 91% sequence homology with *Desulfovibrio desulfuricans*. These two bacteria were distinguished, however on the basis of further genetic (rDNA analysis involving other members of the *Desulfovibrionaceae*), phenotypic (sulphate-reduction assay, electrophoretic protein profiles) and life cycle differences (McOrist et al., 1995a).

Attempts to culture *L. intracellularis* on conventional media designed for growth of *D. sulfuricans* proved unsuccessful (Collins et al., 1996; McOrist et al., 1995a). Indeed, *L. intracellularis* has not been cultured on any cell-free medium to date, and this greatly hindered the initial identification of the cause of the disease. The establishment of an *in vitro* cultivation system has proved vital in the study of the organism and its habitat (Stills, 1991; Lawson et al., 1993).

The organisms are curved bacilli, approximately 1.5μm in length and 0.3μm in width (McOrist et al., 1995a). They are non-motile, and while no fimbriae or pili have been observed, a long, single, unipolar flagellum has been seen via electron microscopy in several isolates (Lawson and Gebhart, 2000). The organisms are microaerophilic, and this may explain their tropism for the crypts within the porcine intestine, where the dissolved oxygen tension is between 5 and 10% (Hillman et al., 1993). *L. intracellularis* has a proven causal role in the disease proliferative enteropathy (PE) with reproducible pig and hamster challenge studies (using pure cultures of bacteria) having demonstrated the consistent presence of the intracellular organism within the apical cytoplasm of proliferating enterocytes (McOrist et al., 1993b; Jasni et al., 1994a and b).

Proliferative enteropathy (PE) is a clinical syndrome resulting in dehydration and weight loss in weanling and young adult pigs (affecting animals from 3-4 weeks to adulthood). The majority of cases resolve naturally within a period of six weeks. *Refs.* The disease affects a variety of animals including hamsters, ferrets, foal and deer, but is most economically significant in the pig, an animal in which it has world-wide prevalence. (With evidence for its existence in North America, Biester and Schwarte, 1931; South America, More's et al., 1985; Europe, Emsbo, 1951; Asia, Redman Chu and Hong, 1973; Africa, Williams and van der Walt, 1994; and Australia, Love et al., 1977).

L. intracellularis multiplies within the apical cytoplasm of crypt epithelial cells principally of the ileum and colon. While actively dividing bacteria were originally only seen within mammalian enterocytes (McOrist et al., 1994a), the host range has now diversified to include avians such as emus (Lemarchand et al., 1997) and ostriches (Cooper et al., 1997a). *L. intracellularis* has been confirmed as the cause of proliferative enteropathy in deer (Cooper et al., 1997b); dog (Leblanc et al., 1993); emu (Lemarchand et al., 1997); hamster (McOrist et al, 1989); horse (Williams et al., 1996); rhesus macaque monkey (Klein et al., 1999); pig (McOrist et al., 1989b); ostrich (Cooper et al., 1997a); and the rabbit (Schoeb and Fox, 1990). In the blue fox (Ericksen et al., 1990), guinea-pig (Elwell et al., 1981) and the rat (Vandenberghe et al., 1985) morphologically similar organisms have been seen in proliferative lesions (but they were not identified further). Recent challenge experiments, however, did show reproduction of PE using pure cultures of *L. intracellularis* in the rat (Collin et al., 1999). In lambs, intestinal lesions with crypt hyperplasia have been seen, but no intracellular bacteria found (Vandenberghe and Hoorens, 1980).

1.7.1 Porcine Intestinal Adenomatosis.

Porcine intestinal adenomatosis (PIA) presents as an uncomplicated proliferative lesion, with visible thickening of the mucosa in the small and sometimes large intestine as a result of increased crypt cell proliferation (Rowland and Rowntree, 1972). In severe cases this proliferation can resemble adenomatous neoplasia hence the incorporation of the term 'adenomatosis'. While PIA presents this basal pathological picture, with little or no inflammation, proliferative haemorrhagic enteropathy (PHE) affords a slightly more complex picture with thickening of the ileum (as PIA) plus bleeding into the lumen. Generally only seen in older pigs (over 4 months), the mucosal surface shows little apparent gross damage, with the exception of the adenomatous thickening, and there are no obvious bleeding points. Congestion of the mucosal blood vessels is seen, and the haemorrhage is thought to originate in the sub-surface capillary bed (Rowland and Rowntree, 1972). Whereas PIA classically presents with poor weight gain, PHE is associated with bloody diarrhoea and/or sudden death following intestinal haemorrhage.

The causative agent of PE, the obligate intracellular bacterium *Lawsonia intracellularis*, is consistently found within the cytoplasm of proliferating enterocytes and is diagnostic for disease. The bacteria can be seen within pig enterocytes from 5 days following challenge with *L. intracellularis,* visible hyperplasia of enterocytes seen from 10 days, and gross lesions from 21 days (McOrist et al., 1994a). The contrast between regions of diseased tissue, and adjacent healthy areas of the intestine can often be quite marked, and disease does not appear to spread beyond the intestine.

Most of the draining lymph nodes do not show any specific significant changes following infection with *L. intracellularis*. Occasionally, however, intracellular bacteria and lesions similar to those seen within the intestine (including glandular elements) have been noted (Emsbo, 1951; Roberts et al., 1980).

1.7.1.1 Detection of *L. intracellularis.*

An epidemiological survey carried out by Smith et al. (1998) indicated that the incidence of proliferative enteropathy in British pig farms was estimated at 31%. The effect of the disease on economic performance was a 23-30% reduction in weight gain, associated with an increased feed requirement of up to 15% (McOrist et al., 1997a). Due to the difficulty in accurately diagnosing PE ante-mortem the ability to monitor the disease in clinical or epidemiological studies is limited, and the precise prevalence and cost to the pig industry can not be exactly determined. PE can be detected post-mortem by gross and histologic lesions and specific detection of *L. intracellularis.* Haematoxylin and eosin staining reveals hyperplasia of crypts sometimes associated with branching, and a reduction in the number of goblet cells (McOrist et al., 1989b). Immunohistochemistry using a specific monoclonal (McOrist et al., 1987) or polyclonal (Lawson et al., 1985) antibody is used to detect the intracellular organisms.

The serological response to *L. intracellularis* will be covered in more detail later (section 1.7.3.1). A serodiagnostic test is commercially available but diagnosis has previously not proven reliable at detecting low level infections (Lawson et al., 1988; Holyoake et al., 1994).

Polymerase chain reaction (PCR) was first used by Jones et al. (1993a) to detect *L. intracellularis* in the faeces of infected pigs. Further work by McOrist et al. (1994b) found the organism in both faeces and intestinal mucosa. Detection of the organism was only possible, however, in animals that were currently suffering with PE.
There was apparently no ability to detect a carrier or sub-clinical state of infection (Smith and McOrist, 1997), indicating that elimination of the bacteria from the gastrointestinal tract would be sufficient to cure an animal of disease.
PCR detection of organisms shed in faeces demonstrated initial detection at 2 weeks post challenge, with infection being detected for at least 10 weeks (Smith and McOrist, 1997). This further supports the idea that the organism has an incubation period of 2-3 weeks (Mapother et al., 1987; McOrist and Lawson, 1989).

1.7.2 Pathogenesis.

1.7.2.1 Transmission/host cell tropism.

L. intracellularis is thought to be transmitted via the faecal-oral route. Once within the intestinal lumen the organisms demonstrate tropism for epithelial crypt cells, where they preferentially invade the rapidly dividing immature enterocytes.

1.7.2.2 Attachment/entry.

Obligate intracellular pathogens must gain access to a permissive host cell before they can multiply and initiate further spread. This may include those normally termed non-phagocytic (entry into phagocytic cells such as macrophages is usually mediated by the process of normal phagocytosis i.e. via broad reactive receptors for mannose and/or fucose, via FcR after immunoglobulin binding or via complement receptors; Kaufmann, 1993).

Different organisms employ different strategies to do this, e.g. the use of integrins as invasin receptors by the enteropathogenic *Yersiniae;* cytoskeletal rearrangements induced by *Salmonella* secreted proteins*;* and novel micro-filament independent pathways by certain strains of *Campylobacter jejuni* and *Citrobacter freundii* (Oelschlaeger et al., 1993).

In vitro attachment studies have shown that prior to internalisation of *L. intracellularis* in an endocytic vacuole at the brush border, the monoclonal antibody VPM53 (specific for *L. intracellularis*), is capable of reducing attachment, suggesting that a surface-associated factor of *L. intracellularis* is involved in the invasion of host epithelial cells (McOrist et al., 1997b). This was thought to be due to the antibody acting directly on a bacterial adhesin rather than creating steric hindrance (McOrist et al., 1997b). *In vivo* studies (Jasni et al., 1994b) also demonstrated entry of bacteria from the lumen following attachment to the microvillous brush border. Further *in vitro* studies demonstrated that cytochalasin D had an inhibitory affect (of approximately 60%) on the internalisation of *L. intracellularis,* indicating that cell entry is likely to be an actin-dependent process involving cell microfilament activity (Lawson et al., 1995), this is also required for *Salmonella* and *Shigella* entry (Finlay and Falkow, 1997). Additional work demonstrated that attachment and entry of *L. intracellularis* into enterocytes was dependent on host cell viability but not bacterial viability (Lawson et al., 1995). This is in contrast to *Salmonella* and *Shigella* species which require host cell activity as well as metabolically active bacteria (Finlay and Falkow, 1989), but similar to *Chlamydia* and *Yersinia*, which do not require bacterial viability for cell entry. (Moulder, 1985).

Salmonella, Shigella and *Listeria,* are all organisms capable of growth and replication within host cells. Whilst these bacteria all vary in the mechanisms employed to enter the cell, they share the fact that they all induce their own uptake pathways. *L. intracellularis* is a unique intracellular pathogen, with as yet undefined methods for cell entry that are apparently quite distinct from previous documented entry pathways for intracellular organisms.

1.7.2.3 Escape from phagosome.

L. intracellularis, along with a variety of organisms such as *Listeria, Shigella, Rickettsia* and *Trypanosoma,* are intracellular pathogens known to destroy the endocytic vacuole following uptake by the host cell, and multiply within the cytoplasm (Finlay and Falkow, 1997). In contrast organisms including *Legionella pneumophila, Mycobacterium tuberculosis* and *Salmonella typhimurium* modify this vacuole and remain within the phagosome (Garcia-del Portillo and Finlay, 1995). Various mechanisms used for survival by pathogens multiplying within the endocytic vacuole include blocking phagosome maturation into a phagolysosome compartment; inhibiting phagosome acidification, thereby preventing optimal activity of acid hydrolases; and adapting to the acidic intravacuolar environment (including developing resistance to acid hydrolases). The escape of intracellular pathogens into the cell cytosol allows the organisms access to the cell nutrients, as well as allowing them to subvert the hosts immune response. Association of *L. intracellularis* with the cell membrane of enterocytes has been seen *in vitro* up until three hours following initial challenge of the cell monolayer (McOrist et al., 1995b), with all bacteria having escaped from the endocytic vacuole in which they were internalised by 24 hours.

1.7.2.4 Multiplication/spread.

In vivo once immature enterocytes have become colonised by *L. intracellularis* they apparently fail to mature and continue undergoing mitosis. This leads to the formation of elongated and branched glands (since the cells are not shed) and the absence of secretory goblet cells (Rowland and Lawson, 1974). Following division of infected cells, the nuclear and cytoplasmic contents, including the intracellular bacteria, are passed on to the daughter cells (Collins et al., 1996; Lawson et al., 1993). The focal pattern of infection in *in vitro* IEC-18 monolayers is consistent with the *in vivo* contrast between diseased proliferating tissue and adjacent areas of healthy epithelium. Further evidence for the spread of infection resulting from division of primary infected cells is that *in vitro* infection is shown not to spread to adjacent cells once the monolayer has reached maturity (Lawson et al., 1993).

In vivo epithelial cells, and organisms, are finally shed into the lumen, following the high degree of turnover of enterocytes, and excreted in faeces.

1.7.3 Immune response to *L. intracellularis*.

The apparent lack of a host cellular response in lesions of proliferative enteropathy, in conjunction with the apparent neoplastic nature of the affected gland cells, contributed to the early suggestions that the disease was either caused by a viral or a non-infective agent (Lawson and Rowland, 1974). Histologically PE has been associated with a weak inflammatory response and little infiltration of either granulocytes or lymphocytes (McOrist et al., 1992).

This is in marked contrast to the induction of a high level of disruption to the normal intestinal milieu; encompassing an inflammatory response of neutrophils, macrophages, and lymphocytes seen by many enteric pathogens. This may extend to congestion, altered GALT and disruption of growth of the microvilli in some instances. During the acute inflammatory response seen in infectious enterocolitis, the intestinal crypt epithelium is infiltrated by neutrophils, which transmigrate to the luminal side of the crypts, this includes *Salmonella, Campylobacter* and *Shigella* (Huang et al, 1996). The initial response generated by mice against *L. monocytogenes*, for example, relies on neutrophil infiltration, and the possible involvement of NK cells (DiTirro et al., 1998). Later responses involve resident macrophages and an influx of bone marrow-derived phagocytes in the liver and spleen (Nomura et al., 1998). Bacilli are released from host cells (parenchymal cells and hepatocytes) following lysis by neutrophils and macrophages with the latter becoming activated to a bactericidal state by IFNγ production. The late response (seen from day 6) involves antigen-specific TCR αβ cells ($CD8^+$ CTLs and IFNγ producing $CD4^+$ T cells). Hiromatsu et al. (1992) proposed a role for γδ T cells in the early response against *L. monocytogenes* with these cells producing macrophage chemotactic and activating factors.

The immune response to proliferative enteropathy has previously been characterised as being a weak humoral and cellular response (McOrist et al., 1994a). There has been little evidence of T cell infiltration, particularly in early lesions. Only in later lesions, probably of several weeks' duration, is an infiltrate of cytotoxic and activated T ($CD8^+$, $CD25^+$) lymphocytes together with IgM^+ B cells evident in the mucosa (McOrist et al., 1992), along with a small number of mononuclear and polymorphonuclear leukocytes. In affected pigs the lymph nodes demonstrated moderate hyperplasia of follicular, interfollicular and medullary lymphoid cells (McOrist et al., 1993). Natural disease can also result in enlargement of the mesenteric lymph nodes, with histologically evident intracellular organisms (Roberts et al., 1980). The documented immune response to date following infection with *L. intracellularis* therefore suggests that the organism induces a unique reaction among enteric pathogens. The analysis of the immune response however, was not performed in a controlled and systematic manner, and frequently relied on the assessment of available data from post-mortem cases.

1.7.3.1 Serology.

The existence of circulating antibody responding to *L. intracellularis* was initially shown by Rowland and Lawson (1974), when they demonstrated that sera from pigs with lesions, when coupled with FITC, recognised the intracellular organisms within the apical cytoplasm of affected enterocytes. This activity, while blocked by the addition of sera from other known PE cases, was not inhibited by sera from a herd free of disease.

Later studies demonstrated that specific IgA and IgM responses were detected both within the serum (Lawson et al., 1988) and the mucosa (Lawson et al., 1979) of infected pigs, but the IgG response was weak (Lawson et al., 1988). The presence of IgA within affected enterocytes (McOrist et al., 1992) may result from an inability of the cell to transcytose the immunoglobulin, either due to the presence of the bacteria, or the cells immaturity (specificity of the immunoglobulin for *L. intracellularis* was not shown).

The IgM response monitored was shown to be short-lived and persisted for approximately eight weeks (McOrist et al., 1992). The majority of animals showing pathological signs of disease at necropsy demonstrated an antibody response (with only a small percentage of cohort animals without lesions found to have serum IgM antibody). Animals exposed to diseased mucosa that did not develop lesions of proliferative enteropathy, also failed to develop an IgM response to *L. intracellularis* (Lawson et al., 1988).

An ELISA established by Holyoake et al. (1994) to look at the presence of IgG generated against *L. intracellularis* concluded that the animals produced low IgG titres, but it was thought that this antibody would play a minor role in the prevention of PE in pigs.

There remains much to be determined about the immune response to *L. intracellularis,* and the development of a suitable animal model will lead to an improved understanding of PE.

1.8 Models of bacterial infections

While a great deal of knowledge about the pathogenic mechanisms of bacteria has arisen from work carried out *in vitro*, there is an undisputed requirement to establish bacterial pathogenicity *in vivo*. Here, environmental conditions vary not only from those seen *in vitro*, but also alter as the infection progresses in response to inflammation, tissue breakdown and spread from one site to another (Smith, 1998). In response to these conditions bacterial behaviour must change to accommodate altered availability of nutrients and substrates, and the subsequent effect this has on the regulation of gene expression. For example, the complement of virulence determinants may change with time and anatomical site of replication. *In vivo* models allow for the demonstration of complex networks of interactions between the various cells and molecules of the immune system that are responsible for protection of the host from pathogenic micro-organisms. Further to this, studies utilising knockout animals (with specific gene deletions) can produce insights into the functions of a modified immune system for example.

Following a specific gene deletion it is possible for there to be a general breakdown in antimicrobial immunity; for the remaining functional aspects of the immune system to compensate for the generated loss; or for there to be a specific and exclusive effect on the host antimicrobial immune response. The reality usually lies somewhere between the latter two examples. This can highlight unique aspects of the immune system required for the elimination of certain organisms (without which the host would succumb to infection). If the response to infection between a fully immunocompetent wild-type animal and one that has been specifically genetically altered is compared it may then be possible to establish the differences between the two situations, and attribute the altered response (whether directly or indirectly) to the missing element of the immune system. Both the direct implications and the potential knock-on effects that the elimination of a specific aspect of the immune system may present should be considered. An apparent lack of a functional defect following genetic manipulation of the hosts' immune system does not mean that that particular aspect of the immune response is not significant in the natural response to infection. Instead it suggests the degree of flexibility, and redundancy, to be found within the immune system. This encompasses a compensatory effect to reduce the influence of the deleted aspect of the immune system. Rarely, however, is completely functional immunity restored. Nevertheless a great deal of knowledge has been obtained from experiments that have utilised model systems. For example understanding of the immune response against *L. monocytogenes* has been greatly advanced by the use of a variety of different knockout animals, that when analysed together, allow a more detailed picture to be presented. Hereby each aspect of the immune system can be assessed in some detail to determine its effect on disease outcome. Macrophages are known to act as key effector cells in protection from listeriosis. The requirement of T cells to activate their antibacterial potential (Mackaness and Hill, 1969), involvement of both $\alpha\beta$ and $\gamma\delta$ T cells, and the inability of B cells to adequately control infection in the absence of functional T cells (Mombaerts et al., 1993a and b) has all been established following the use of various mice with specific gene deletions.

Other studies have demonstrated that mice deficient in interleukin six (IL-6) show an increased susceptibility to experimental listeriosis, in part due to the reduced activation of an inflammatory response (and it may also be due to the effect that IL6 has on the development of B cells).

1.8.1 IFNγR$^{-/-}$.

It is widely accepted that a strong Th1 cell response is required in protection from intracellular bacterial infections (in preference to that generated by Th2 cells), and that IFNγ, partly in its role as a macrophage-activating factor, is an important product of these activated cells. Analysis of this area of the immune system can therefore offer a great deal of insight into the immune response generated against *L. intracellularis.*

Huang et al. (1993) document the development of an IFNγR$^{-/-}$ mouse on a 129/Sv/Ev background which showed that the immune system of the interferon gamma receptor knockout mice appeared to develop normally, and they were still capable of generating a specific immune response. A defect in natural resistance to challenges with *Lt. monocytogenes* and vaccinia virus was demonstrated however, despite normal cytotoxic and T helper cell responses. They also illustrated that a functional IFNγR was required for a normal antigen-specific IgG2a response, but not a specific T cell response. Kamijo et al. (1993b) showed that in IFNγR$^{-/-}$ mice other available cytokines (such as TNF involvement in the induction of $NO2^-$, the end product of nitric oxide generation) were unable to fully compensate for the absence of functional IFNγ in macrophage activation. Mice with deletions in the IFNγ receptor suffer severely from various intracellular infectious agents including *L. monocytogenes, M. bovis* BCG, *Leishmania major* and African trypanosomes (Huang et al., 1993; Kamijo et al., 1993a and b; and Mabbott et al., 1998). One of the most significant alterations in the immune response as a result of the lack of a functional IFNγR is the inability to activate a protective macrophage response due to altered nitric oxide production (Huang et al., 1993).

Work by Swihart et al. (1995) demonstrated that following challenge of IFNγR$^{-/-}$ mice with *Ls. major* the host mounted a polarised IFNγ producing Th1-type response, without evidence for the expansion of IL-4-producing Th2 cells. This indicates that IFNγ mediated signals are not necessary for the differentiation of CD4^{+} T cell precursors towards a Th1 phenotype. There is thought to be an important role for cytokines present at the initiation of primary CD4^{+} T cell stimulation on the differentiation of naïve CD4^{+} T cells towards one or the other functional phenotype (i.e. Th1 or Th2). IL-4 and IL-12 are known to be involved in the development of Th2 and Th1 responses, respectively. The role of IFNγ in the promotion of Th1 cells is unclear however, although the ability of IFNγ to selectively inhibit the proliferation of Th2 cells *in vitro* has been reported (Gajewski et al., 1989). The results of Swihart et al. (1995) do not support a critical role for IFNγ in Th1 development *in vivo*. They do support the hypothesis that in the absence of sufficient amounts of IL-4, the development of an IFNγ-producing CD4^{+} T cell response could represent the 'default' pathway of differentiation of CD4+ T cell precursors (Seder et al., 1992).

Activated Th1 cells leads to the development of a protective cellular immune response rather than the activation of the humoral aspect of the immune response by Th2 cells. IFNγR$^{-/-}$ mice also have a greatly reduced rate of production of the cytokines TNFα, IL-1 and IL-6, all proinflammatory cytokines (Kaufmann and Ladel, 1994).

1.9 Aim of Research

There is currently little understanding of the immune response generated in response to *L. intracellularis*. Utilising the benefits of animal models (both wild-type animals and those with specific gene deletions) will greatly aid in the process of determining mechanisms of protective immunity. Therefore, the work set out in this thesis aimed to develop an animal infection system to study the pathogenesis and immune response following infection with the novel bacterium *Lawsonia intracellularis*. The study involved the development and assessment of a mouse infection system for proliferative enteropathy using 129/Sv/Ev mice.

The development of a suitable model for infection with *L. intracellularis* will improve our ability to dissect the pathogenic and immunological mechanisms associated with infection by this intriguing intracellular bacterium through characterisation of cellular and humoral aspects of the hosts defences using immunohistochemical analysis and western blot studies. Further to this isogenic 129/Sv/Ev IFNγR$^{-/-}$ mice allowed analysis of the differing aspects of the immune response apparent following infection, by investigating the effect of a specific gene deletion on the immune response particularly involving cells involved in the Th1-pathway in response to this intracellular pathogen.

2. MATERIALS AND METHODS.

All reagents used in this work were supplied by Sigma unless otherwise stated in the text. All company addresses are listed in Appendix I.

Experimental challenge studies have incorporated the use of a variety of wildtype virulent strains of *Lawsonia intracellularis* to successfully reproduce lesions of proliferative enteropathy in several different animals, mainly focusing on pigs and hamsters. These include 916/91 (hamsters, Jasni et al., 1994a; pigs, McOrist et al., 1993; and rats, Collins et al., 1999); 1482/89 (pigs, McOrist et al., 1993); LR189/5/83 (pigs, Smith and McOrist, 1997); N343 (pigs, Knittel et al., 1998), 15540 (pigs, M. Roof, personal communication) and 2189/94 and 253/85 (pigs, Collins et al., 1999). Two of these wild-type strains were used in this challenge study to inoculate both 129/Sv/Ev wild-type and isogenic IFN$\gamma R^{-/-}$ mice.

2.1 Maintenance of *Lawsonia intracellularis in vitro.*

Lawsonia intracellularis was maintained in cell culture using described methods (Knittel and Roof, 1999; Lawson et al., 1993). The IEC-18 cell line (European Cell Culture Collection number 88011801) was derived from normal epithelial cells of the rat ileum and supports growth of *L. intracellularis* without apparent vacuolation or disruption to the normal cell cycle (Lawson et al., 1993). The IEC-18 cells were grown in Dulbecco's modified Eagles medium (DMEM, GibcoBRL) with 10% v/v FCS (plus 1% l-glutamine and 2$\mu g.ml^{-1}$ amphotericin B).

Growth of this fastidious organism to sufficient numbers for challenge inocula was an extremely lengthy and time-consuming procedure and acted as a severe limiting factor in the number of possible experiments.

2.1.1 Trypsinisation.

Uninfected flasks of IEC-18 cells (at 100% confluence) were trypsinised, and seeded to fresh daughter flasks. The media was removed aseptically from the flask, and the confluent monolayer washed with approximately 1ml Versene (GibcoBRL). 5mls Trypsin (GibcoBRL) was added and left at room temperature for 90 seconds. This was removed, and the flask placed in a 5% CO_2, 37°C incubator for 5-10 minutes. Once the cells had detached from the flask, 5mls of fresh medium (plus supplements) was added and the cell suspension dispersed by mixing. A Neubauer chamber haemocytometer was used to calculate the cell number in the suspension. This was adjusted so that fresh tissue culture flasks were seeded with epithelial cells at a concentration of $0.5x10^5ml^{-1}$. Tracs, (plastic bijoux, Bibby sterilin 129AX/1, containing 13mm diameter coverslips) were treated in parallel. The required volume of medium was then added to the appropriate tissue culture flask (5mls for $25cm^2$, 15mls for $75cm^2$ and 30mls for $150cm^2$). All flasks were maintained in a 5% CO_2 atmosphere at 37°C. Cell adherence, presence of contamination, and colour of phenol red indicator (medium) were regularly monitored. The cells reached 20% confluence overnight, prior to infection with *L. intracellularis.*

2.1.2 Infection with *L. intracellularis*.

To maintain the growth of *L. intracellularis* within the IEC-18 cell line the organisms were passaged on a weekly basis. The supernatants from established infected flasks were aseptically decanted and 2-5mls of sterile hypotonic Potassium chloride (KCl, 0.1% w/v) added to the confluent monolayer. This was incubated at 37°C for 4-10 minutes (variation in time due to fact that older cell monolayers i.e. higher passage number of epithelial cells, as well as heavily infected cells became refractile quicker). The 0.1% KCl was decanted and replaced with 2-5mls SPG (Sucrose:0.218M, KH_2PO_4:0.0038M, K_2HPO_4:0.0072M, Potassium glutamate:0.0049M) and the cells resuspended using a cell scraper. The cell membranes were lysed by passing the suspension through a Luer Lock™ 20g needle three times.

The lysed cell suspension was centrifuged at 12000rpm for 7 minutes (in an MSE Mistral 1000), the cell nuclei removed, and the supernatant added to warmed medium (DMEM, 7%FCS, 2mM l-glutamine, $2\mu g.ml^{-1}$ amphotericin B and $100\mu g.ml^{-1}$ vancomycin). Fresh flasks and Tracs were inoculated with volumes appropriate to the flask size (as before). The flasks were transferred to an anaerobic jar, evacuated to -500mmHg, replaced with hydrogen, and maintained in a microaerophilic atmosphere, 8% O_2, 8.8% CO_2. The cultures were refed with fresh medium at days 2 and 5. On day 7 the monolayers were 100% confluent and the process of cell lysis and passage was repeated until sufficient inocula was generated. The concentration of organisms inoculated into the fresh flasks was calculated following specific immunohistochemical staining of the parallel infected Tracs, as detailed below.

2.1.3 Monitoring of infection in cell culture.

Concurrent with seeding of the appropriate tissue culture flasks, Tracs were set up to monitor the development of infection. At day 5 following infection the coverslips from Tracs were harvested and immunostained as follows: using mouse monoclonal antibody VPM53 (prepared in house, McOrist et al., 1987) specific for *L. intracellularis*. The Tracs were immunostained using an immunoperoxidase technique and a mouse monoclonal antibody (VPM53). The coverslip was removed from the Trac bottle and washed in phosphate buffered saline (PBS; 137mM NaCl, 27mM KCl, 8mM Na_2HPO_4, 15mM KH_2PO_4, pH 7.2) for 5 minutes, then placed in acetone for 2x30 seconds. Once removed and dried, they were fixed to a glass slide using a drop of Loctite™ glass bond glue, and set by exposure to UV light. Staining was carried out in a moisture chamber: firstly slides were washed in PBS for 5 minutes, then for 2x10 minute washes with PBS-0.5% azide (to block endogenous peroxidase). Next a 5 minute wash with PBS, preceded 10 minutes with IPX buffer (0.08% Tween 80, 0.1% BSA in PBS). The slides were incubated with the primary mouse monoclonal antibody (VPM53), diluted 1:200 in IPX buffer, for 30 minutes at 37°C. The slides were rinsed in PBS, washed for 5 minutes in IPX buffer, and incubated with the secondary antibody (goat anti-mouse HRP) at room temperature for 1 hour.

Slides were then rinsed in IPX buffer and PBS (for 5 minutes each) before the DAB substrate (3,3'-diaminobenzedine, Sigma) was added for 5 minutes. Finally the slides were counterstained with haematoxylin and mounted in DePeX (BDH Merck). *L. intracellularis* stained deep brown, and the nuclei of the epithelial cells blue/purple.

Bacterial proliferation was assessed by calculating the number of specifically stained bacteria associated with cells in a unit area of the coverslip (Lawson et al., 1993). The number of epithelial cells infected with *L. intracellularis* was counted, and bacterial numbers estimated by extrapolation. Growth of *L. intracellularis* was maintained in cell culture over many passages, and sufficient organisms for *in vivo* challenge studies were grown using this methodology. This time-consuming process required 4-6 weeks to cultivate a bacterial suspension of approximately $1x10^8$ organisms.ml^{-1}. In the experimental protocol used this was sufficient for approximately 16 animals. Verification of the absence of bacterial contaminants was confirmed as described by Lawson et al. (1993).

2.2 *In vivo* infection system.

Wild-type 129/Sv/Ev mice and interferon gamma receptor null ($IFN\gamma R^{-/-}$) 129/Sv/Ev mice (Huang et al., 1993), were purchased from Bantum and Kingman Universal, Hull, and bred in-house. Mice were challenged by oral gavage (0.25-0.3ml), via a steel ball ended canula attached to a 1ml syringe. The canula was placed in the proximal oesophagus to ensure that the inocula reached the stomach. Mice were inoculated with either SPG buffer alone, or a suspension of *L. intracellularis* (as detailed below). (K11 and K18 indicate the inoculum has been passaged through the weekly cell culture procedure eleven and eighteen times respectively. SN6 indicates that the inoculum was taken from the supernatant of heavily infected tissue culture flasks after 5 weekly passages).

Tables 2.1 and 2.2 illustrate the four different challenge studies undertaken, the groups of mice used in each study, the dose given, and the age of animals at time of inoculation.

Experiment	*L. intracellularis* dose	Age at challenge
1	$5x10^6$ per animal (SN6)	38-66 days
2	$5.5x10^7$ per animal (K11)	58-84 days
3	$5.5x10^7$ per animal (K11)	47-62 days
4	$1.9x10^7$ per animal (K18)	59-94 days

Table 2.1. Challenge dose and age of mice at the time of inoculation.

	Days post inoculation						
	7	**14**	**21**	**28**	**35**	**40**	**Total**
Expt.1							
WT	4	4	4	4		2	18
WT C			2				2
IFNγR$^{-/-}$	4	4	4	4		2	18
IFNγR$^{-/-}$C			2				2
Expt.2							
WT		4	4	4	4		16
WT C		2	2	2	2		8
IFNγR$^{-/-}$		4	4	4	4		16
IFNγR$^{-/-}$C		2	2	2	2		8
Expt.3							
WT		4	4	4	4		16
WT C		2	2	2	2		8
IFNγR$^{-/-}$		4	4	4	4		16
IFNγR$^{-/-}$C		2	2	2	2		8
Expt.4							
WT		10	10	10			30
WT C		5	5	5			15
IFNγR$^{-/-}$		10	10	10			30
IFNγR$^{-/-}$C		5	5	5			15

Table 2.2. Summary of groups for the four challenge experiments.

The table illustrates the number of mice in each group dosed with *L. intracellularis* (WT, wild-type or IFNγR$^{-/-}$) and control (C), and the number of days post-inoculation until necropsy.

2.2.1 Necropsy procedure.

At necropsy the abdominal cavity was opened and the entire gastrointestinal tract was removed. The tract was dissected and separated on a dissection board. The intestine was opened longitudinally and the mucosal surface examined for evidence of gross lesions. Regions of the gastrointestinal tract were then prepared by twisting them in concentric centrifugal circles round a plastic Pasteur pippette, with the serosal side innermost to create a 'swiss roll', allowing a greater section of the gastrointestinal tract to be examined (Moolenbeek and Ruitenberg, 1981).

2.2.1.1 Paraffin-embedded tissue.

Sections of ileum and colon were either fixed in 10% phosphate buffered formalin (BDH Merck) for a minimum of 24 hours or fixed for a minimum of six hours in 4% paraformaldehyde (in PBS) and then stored in 70% ethanol until processing. All tissue samples were processed for 4 hours in a Behr VIP 2000, where they were dehydrated though graded alcohols, cleared in xylene and then impregnated in paraffin wax.

2.2.1.2 Snap frozen tissue.

Where appropriate, samples of spleen, ileum, colon and mesenteric lymph nodes were embedded in Tissue-tek O.C.T. compound and frozen in a dry ice/isopentane slurry (BDH Merck) and stored at -70°C until required.

2.2.1.3 Electron microscopy.

Samples taken for analysis by electron microscopy were fixed for 2-3 hours (3% gluteraldehyde in 0.1M Sodium Cacodylate Buffer), then washed for 3x20 minutes in 0.1M sodium cacodylate buffer. This was followed by a post-fixation step of 1 hour in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, then the tissue was stored in 0.1M sodium cacodylate buffer at –4°C until required. Subsequently all samples were dealt with in the Electron Microscopy Suite by Mr. S.Mitchell.

Briefly, the tissue was washed for 3x20 minutes in distilled water and dehydrated through graded acetone (50% acetone for 10 minutes; 70% acetone for 10 minutes; 90% acetone for 10 minutes; and finally 100% acetone for 3x10 minutes). The tissue was embedded in Araldite^{TM} (Agar Scientific Ltd), and 60nm sections cut on a diamond knife using a Reichert OMU4 Ultracut Ultramicrotome, and mounted on mesh copper grids.

The sections were stained with uranyl acetate and lead citrate using an LKB Ultrostainer. Sections were viewed and photographed using a Philips 400 transmission electron microscope in the Faculty of Veterinary Medicine EM suite.

2.2.1.4 Blood sampling.

Heparinised blood samples were collected from mice (from Experiment 3 immediately prior to necropsy, and serially at seven day intervals for Experiment 4) by drawing blood into a heparin-coated syringe (1000U/ml^{-1}, Leo Laboratories Ltd). The samples were centrifuged at 10,000g for 15 minutes, the plasma removed, and kept frozen at –20°C until required.

2.2.2 Experiment One.

A total of 18 WT and 18 IFNγR$^{-/-}$ mice of 38-66 days old were challenged with approximately 5x10^6 *Lawsonia intracellularis* LR189 (SN6, in 0.5ml). Two WT and IFNγ mice were inoculated with SPG only as controls (culled at day 21). At days 7, 14, 21, and 28 post inoculation four mice from each background were culled and necropsied. At day 40 post inoculation the two remaining inoculated mice were culled (Table 2.2).

All mice were euthanased by a Schedule One method. The average weight of each group was monitored before gavage, and again at post-mortem.

2.2.3 Experiment Two.

WT and IFNγR$^{-/-}$ mice were challenged with a 0.25ml suspension containing either *L. intracellularis* LR189 (5.5x10^7 bacteria) or SPG only (as a control). 16 inoculated and 8 control mice were in each of the two strain groups.

At 14, 21, 28 and 35 days post-inoculation four inoculated, and two control, mice from each group were euthanased by a Schedule One method and necropsied. Sections of colon and ileum were removed and fixed in buffered formalin.

2.2.4 Experiment Three.

WT and IFNγR$^{-/-}$ mice were challenged with 0.3ml of *L. intracellularis* LR189 ($5.5x10^7$ bacteria) or SPG as a control. There were 16 challenged and 8 control mice in each of the two strain groups. Animals were euthanased by a Schedule One method at 14, 21, 28 and 35 days post-inoculation in groups of 6 (4 inoculated and 2 control mice per group). This was preceded by bleeding via the tail vein. At necropsy samples of ileum and colon were frozen in a dry-ice/isopentane slurry, as well as sections removed for formalin-fixation, and also for processing for electron microscopy. MLN and spleen samples were also collected and snap frozen.

2.2.5 Experiment Four.

WT and IFNγR$^{-/-}$ mice were challenged with 0.3ml of *L. intracellularis* 15540 ($1.9x10^7$ per animal) or SPG as a control. There were 30 challenged and 15 control mice in each of the two strain groups. Animals were euthanased by a Schedule One method at 14, 21 and 28 days post-inoculation in groups of 15 (10 inoculated and 5 control mice).

At necropsy samples of ileum and colon were frozen in a dry-ice/isopentane slurry, as well as sections of ileum and colon fixed in 4% para-formaldehyde overnight, then maintained in 70% methanol prior to processing.

2.3 Histopathology.

2.3.1 Haematoxylin and Eosin.

4μm tissue sections of ileum and colon were cut from paraffin embedded wax blocks on a microtome (Leica RM2155), dried onto glass slides, and stained with haematoxyiin and eosin. Staining was carried out using the Shandon Linistain GLX. Slides were dewaxed in xylene, hydrated through graded alcohols, and washed in tap water. Nuclei were stained with Harris haematoxylin (Surgipath) and then washed in tap water.

Slides were washed in Scott's tap water substitute (7g sodium bicarbonate, 40g magnesium sulphate, dissolved in 2 litres of tap water), tap water, and counterstained in Putts Eosin (5g eosin, BDH, 2.5g potassium dichromate, 50mls saturated aqueous picric acid, 50mls absolute ethanol, 50mls distilled water). Finally the sections were washed in tap water, dehydrated in absolute ethanol, cleared in xylene and mounted in DePeX.

2.3.2 Immunohistochemistry.

All immunohistological staining was carried out on either polylysine coated glass slides (BDH) or glass slides previously treated with tissue adhesive (Biobond, British BioCell International). Slides for Biobond treatment were washed overnight in a 5% solution of Decon, rinsed thoroughly with tap water, and air-dried. Slides were then placed in acetone for 4 minutes, followed by 2% solution of Biobond (in acetone) for 4 minutes. Finally they were rinsed for 5 minutes in distilled water, covered and left to air-dry before storage in a dust free environment. Tissue sections were cut from paraffin embedded wax blocks at 4μm on a microtome, mounted, dried at 60°C for 30 minutes, and then 37°C overnight (and kept until required). Alternatively frozen sections were cut at 6μm on a cryostat (Shandon 620M), mounted and either stored at –70°C, or immediately air-dried for a minimum of 1-2 hours at room temperature prior to staining. Slides were then fixed in acetone for 2 x 10 minutes, with air-drying between and after the fixation steps. The slides were then used for immunohistochemical staining immediately. Table 2.3 lists the antibodies used and their sources.

Clone	Antigen	Source
	CD3	Dako
CT-CD4	CD4	Serotec
	CD8	Serotec
5C6	CD11b	Serotec
M290	CD103	BD PharMingen
GL3	γδ TCR	Serotec
C-19	E-cadherin	Santa Cruz Biotechnology Inc.
VPM53 or 1080/76	*L. intracellularis*	In house
RA3-6B2	B220	Serotec

Table 2.3. List of antibodies used in immunohistochemical staining (and their sources).

2.3.2.1 Detection of *L. intracellularis.*

Lawsonia intracellularis was detected by immunohistochemical staining using either rabbit polyclonal antibody, 1080/76 (Lawson et al., 1985) or the mouse monoclonal antibody VPM53 (McOrist et al., 1987), on sections cut from wax-embedded tissue.

For the rabbit polyclonal antibody the commercial Vectastain ABC kit (rabbit) from Vector laboratories was used, and the slides were stained following the manufacturer's instructions. Briefly, the slides were dewaxed in xylene and endogenous peroxidase blocked using 0.06% hydrogen peroxide (BDH Merck) in methanol for 30 minutes. The slides were trypsinised (30 minutes at 37°C (ICN trypsin, 0.1% CaCl), blocked using normal serum (30 minutes) and incubated with the primary antibody (1 hour at 37°C). This was followed by incubation with the secondary biotinylated link reagent (30 minutes) and the ABC complex (30 minutes). Finally visualisation used DAB as the substrate (Vector Laboratories). The slides were counterstained with haematoxylin and mounted in DePeX before being viewed under the light microscope.

Alternatively identification of *L. intracellularis* was assessed using a Mouse on Mouse (M.O.M) kit from Vector laboratories which utilised the mouse monoclonal antibody VPM53 specific for *L. intracellularis* (McOrist et al., 1987). Briefly, the slides were dewaxed in xylene, rinsed for 5 minutes in tap water, and endogenous peroxidase blocked using 3% hydrogen peroxide (BDH Merck) in water for 5 minutes. The slides were trypsinised (20 minutes at 37°C (ICN trypsin, 0.1% CaCl), blocked using a mouse IgG blocking reagent for 1 hour. The slides were then washed in PBS, incubated with M.O.M Diluent for 5 minutes before addition of the primary antibody at 1/200 (30 minutes at room temperature). Incubation with the biotinylated anti-mouse IgG reagent (10 minutes) and the ABC complex (5 minutes) followed. Finally visualisation used DAB as the substrate (Vector Laboratories). The slides were counterstained with haematoxylin and mounted in DePeX before being viewed under the light microscope.

2.3.2.2 Immunohistochemical staining specific for CD11b.

The CD11b antibody (Serotec) was used on frozen tissue sections. Following slide fixation endogenous peroxidase was blocked for 20 minutes using Dako endogenous peroxidase blocker. The slides were treated with 5% normal mouse serum (NMS)/PBS for 30 minutes, then incubated with the primary antibody (1/100) for 1 hour. This was followed by a PBS wash, then incubation with the secondary antibody (biotinylated mouse anti-rat, Sigma at 1/500) for 30 minutes. Then sections were rinsed in PBS, streptavidin POD (Boehringer Mannheim) added for 30 minutes (1/1500), and visualised using DAB (Sigma). The slides were counterstained using haematoxylin, mounted in DePeX, and viewed under the light microscope.

2.3.2.3 Immunohistochemical staining specific for CD3.

The presence of CD3 was detected by the immunohistochemical staining of slides cut from wax-embedded tissue using a Dako rabbit anti-human primary antibody that demonstrated strong cross-reactivity (Jones et al., 1993b; and Dako laboratories). A commercial Vectastain ABC kit (rabbit) from Vector laboratories was used and the slides were stained following the manufacturer's instructions. Briefly, the sections were dewaxed in xylene and endogenous peroxidase was blocked using 0.06% hydrogen peroxide (BDH Merck) in methanol for 30 minutes. The sections were microwave treated in 0.1M citrate buffer in a pressure cooker for 4 minutes, then blocked using normal serum (30 minutes) and incubated with the primary antibody (1 hour). This was followed by incubation with the secondary biotinylated link reagent (30 minutes) and the ABC complex (30 minutes). Finally visualisation used DAB as the substrate (Vector Laboratories). The slides were counterstained with haematoxylin and mounted in DePeX before being viewed under the light microscope.

2.3.2.4 Immunohistochemical staining specific for CD4 and CD8.

CD4 and CD8 antibodies (Serotec) were used on frozen tissue sections. Endogenous peroxidase was blocked for 20 minutes using Dako endogenous peroxidase blocker. The slides were treated with 5% normal mouse serum (NMS)/PBS for 30 minutes then incubated with the primary antibody at the appropriate dilution overnight at room temperature. (The antibodies were titrated and the biotinylated CD4 used at 1:20 and the CD8 at 1:800). Those sections with CD8 as the primary antibody were washed in PBS and incubated for 2 hours with the secondary antibody (mouse anti rat biotin labelled, 1/500 in 1% NMS in PBS). Then both CD4 and CD8 stained sections were rinsed in PBS, streptavidin POD (Boehringer Mannheim) added for 1 hour (1/1500), and visualised using DAB. The slides were counterstained using haematoxylin, mounted in DePeX, and viewed under the light microscope.

2.3.2.5 Immunohistochemical staining specific for CD103.

The CD103 antibody (Serotec) was used on frozen tissue sections. Following slide fixation endogenous peroxidase was blocked for 20 minutes using Dako endogenous peroxidase blocker. The slides were treated with 5% normal mouse serum (NMS)/PBS for 30 minutes, then incubated with the primary antibody (1/100) for one hour. This was followed by a PBS wash, then incubated with the secondary antibody (biotinylated mouse anti-rat, Sigma at 1/500) for 30 minutes. Then sections were rinsed in PBS, streptavidin POD (Boehringer Mannheim) added for 30 minutes (1/1500), and visualised using DAB (Sigma). The slides were counterstained using haematoxylin, mounted in DePeX, and viewed under the light microscope.

2.3.2.6 Immunohistochemical staining specific for E-cadherin.

The E-cadherin antibody (Santa Cruz) was used on paraffin-wax embedded sections. Briefly, the sections were dewaxed in xylene and endogenous peroxidase was blocked using 1% hydrogen peroxide (BDH Merck) in methanol for 30 minutes.

The sections were microwave treated in 0.1M citrate buffer in a pressure cooker for 4 minutes, then blocked using normal rabbit serum (30 minutes) and incubated with the primary antibody (dilution of 1 in 50 for 1 hour). The slides were then washed in PBS and incubated with the secondary antibody (rabbit anti-goat HRP, Dako, at 1/500) for 1 hour. The sections were then rinsed in PBS and visualised using DAB (Sigma). The slides were counterstained using haematoxylin, mounted in DePeX, and viewed under the light microscope.

2.3.2.7 Immunohistochemical staining specific for γδ TCR.

The γδ TCR antibody (Serotec) was used on frozen tissue sections. Following slide fixation endogenous peroxidase was blocked for 20 minutes using Dako endogenous peroxidase blocker. The slides were treated with 5% normal goat serum (NMS)/PBS for 30 minutes, then incubated with the primary antibody (1/20) for one hour. This was followed by a PBS wash, then incubated with the secondary antibody (biotinylated goat anti-hamster, Dako at 1/200) for 30 minutes. Then sections were rinsed in PBS, streptavidin POD (Boehringer Mannheim) added for 30 minutes (1/1500), and visualised using DAB (Sigma). The slides were counterstained using haematoxylin, mounted in DePeX, and viewed under the light microscope.

2.3.2.8 Immunohistochemical staining specific for B220.

The B220 antibody (Serotec) was used on frozen tissue sections. Following slide fixation endogenous peroxidase was blocked for 20 minutes using Dako endogenous peroxidase blocker. The slides were treated with 5% normal mouse serum (NMS)/PBS for 30 minutes, then incubated with the primary antibody (1/300) overnight at 4°C. This was followed by a PBS wash, then incubation with the secondary antibody (biotinylated mouse anti-rat, Sigma at 1/500) for 30 minutes. Then sections were rinsed in PBS, streptavidin POD (Boehringer Mannheim) added for 30 minutes (1/1500), and visualised using DAB (Sigma). The slides were counterstained using haematoxylin, mounted in DePeX, and viewed under the light microscope.

2.3.2.9 Methyl Green-Pyronin staining.

Although not an immunohistochemical stain this technique stains both nucleic acids and was used to distinguish plasma cells in regions of the gastrointestinal tract more clearly due to their densely RNA packed cytoplasms. Briefly paraffin-embedded sections were dewaxed, and then rinsed in distilled water. Methyl green pyronin solution (0.072% aqueous methyl green and 0.57% pyronin Y solution, mixed with an equal volume of acetate buffer solution, pH4.8 BDH) was added for approximately 10 seconds, until the RNA stained red but before the nuclei turned purple. The sections were then rinsed in water, followed by an acetone/xylene mix (50:50), then mounted and viewed under the light microscope.

2.3.2.10 Image Analysis.

The morphometric procedures for counting lamina propria cells involved analysis of immunohistochemically stained slides with a microscope (Leica) and video camera. Digitised images were transferred to a computer and cell counts were performed with a computer image analysis system (Image-Pro Plus 4.1). Ten random areas of colonic tissue were selected, and a specific area of interest delineated on the computer screen within each field (excluding epithelium), see Figure 2.1. Positively stained cells within each region were then counted, and the average for each sample of tissue expressed as positive cells per mm^2. Intraepithelial leukocytes were determined by counting positive cells in crypt epithelial regions, and results were expressed as positive cells per 100 enterocytes.

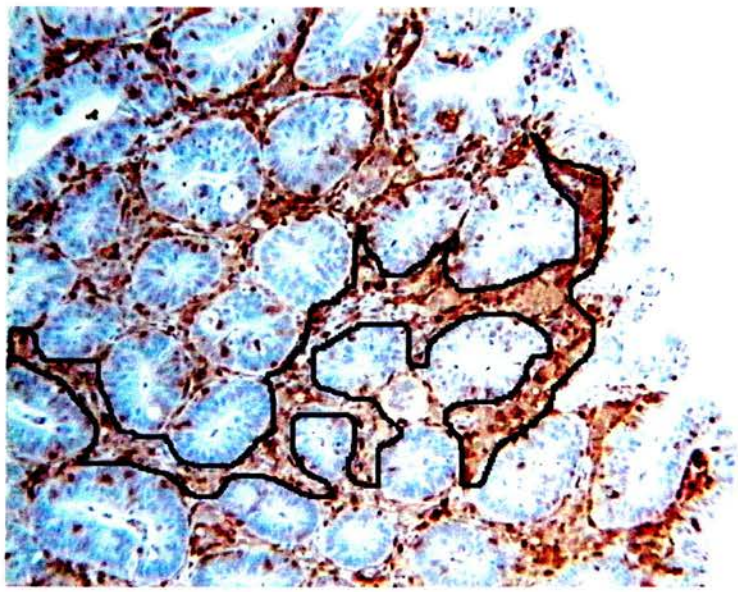
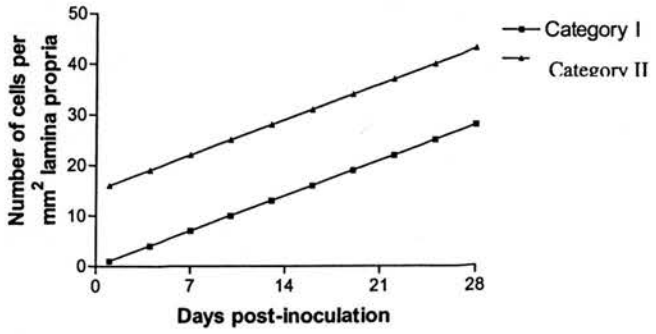


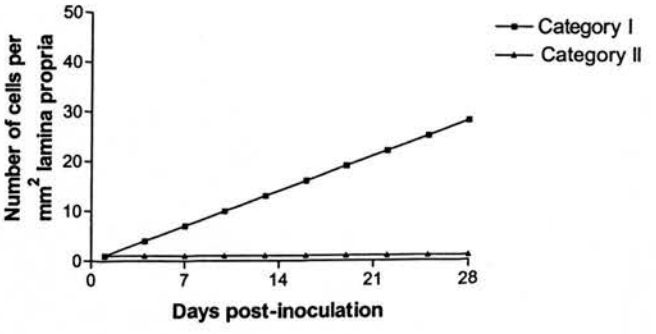
Figure 2.1. Section of colon from an IFNγR$^{-/-}$ mouse 21 days following inoculation with *L. intracellularis*. Following immunohistochemical staining to detect CD3^{+} cells the section of tissue is analysed by marking a specific area (as shown) and counting the number of positive cells per mm^2 lamina propria.

2.3.2.11 Statistical analysis.

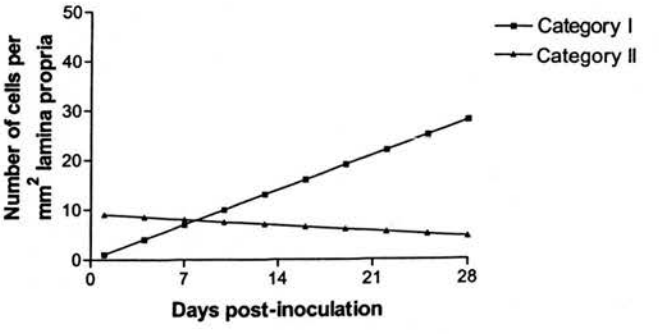
In order to determine significant differences in the relationship between the infiltration of immunohistochemically stained cells and time for control and inoculated groups (for both the WT and IFNγR$^{-/-}$ backgrounds) standard multiple linear regression analyses were carried out using unbalanced general linear models. All analyses were performed using MINITAB version 13.30 and in all cases a $p<0.05$ was taken to indicate significance. In all cases whether linear regression on the parameters (e.g. CD8 infiltration for WT and IFNγR$^{-/-}$ mice) was appropriate was ascertained by checking whether the residuals from the general linear model (GLM) were normally distributed. With the exception of the data for enterocytes IFNγR$^{-/-}$, CD8 WT, CD103 IFNγR$^{-/-}$ and CD11b WT the residuals were normally distributed in all cases. For these four examples the residuals were log normally distributed. Therefore for these parameters GLM was performed on log data. For all four parameters logging the data did not alter the significance of the analyses (i.e. whether the p values were less than, or greater than, 0.05). The aim of the analysis was to establish whether there was significant variation in cell infiltration between the inoculated and control groups of mice with the progression of time. In order to help interpret the results of this procedure Figure 2.2 schematically illustrates the implication of possible differences in the slope and intercept estimated from a linear model. The intercept represents the point at which the line crosses the y-axis at x=0, and is an indication of cell numbers. The slope represents the changes in the cell population over time i.e. a horizontal line indicates no change in cell numbers over the 28 days studied. The null hypothesis states that there will be no change in the number of cells, or rate of cellular infiltration, between the inoculated and control groups over the period of study. In Figure 2.2a the intercepts are different, but the slopes are similar. This implies that there is a similar change in the degree of cell infiltration over the time course studied for the two populations, and that Category I is associated with a greater number of cells per mm^2 lamina propria than Category II. In Figure 2.2b the slopes of the lines are different with Category I again associated with a consistently greater degree of cell infiltration than Category II.



(a)



(b)



(c)

Figure 2.2. Illustrative examples of the output from various general linear models using two hypothetical factors - Category I and II.
(a) different intercept, but similar slope values;
(b) similar intercept, but different slope values;
(c) different values for both the intercept and the slope.

However in this example the intercepts are the same indicating that the actual number of infiltrating cells was the same when x=0 (but that thereafter the cell populations change in a different manner). In Figure 2.2c neither the intercept nor the slope are similar between Categories I or II.

As an example Table 2.4 presents the general linear model for CD3 infiltration in WT mice in control and inoculated animals over the challenge study. The model calculates how much the observed variation in cellular infiltration is described by (a) the progression of time; (b) whether the animals were inoculated with *L. intracellularis* (intercept); and (c) changes in the value of cellular infiltration between animals inoculated with *L. intracellularis* (or not) as time progresses (slope).

	D.o.f.	Sum of Squares	Mean Squares	F value	p value	
Time	1	974.72	518.22	15.32	**<0.001**	(a)
Inoculation?	1	1136.51	793.14	23.45	**<0.001**	(b)
Time*Inoculation	1	400.48	400.48	11.84	**0.001**	(c)
Residuals	41	1386.75	33.82			

Table 2.4. General linear model output assessing the degree of cellular infiltration in association with inoculation of *L. intracellularis* and the progression of time. D.o.f. – degrees of freedom; p value – significance associated with the calculated F value and degrees of freedom. p values of less than 0.05 are shown in bold.

This analysis aims to determine whether the slope of cellular infiltration (c) and/or the intercepts (b) differ significantly ($p<0.05$), therefore the first p value (a) will not be presented in subsequent analyses, and instead only the significance values associated with (b) and (c) will be considered.

WT and IFN$\gamma R^{-/-}$ values were compared using standard 2 sample *t*-tests at a particular time point for the various markers of cellular infiltration.

To determine whether the percentage of crypts infected with *L. intracellularis* was significantly linked with the course of time over the challenge study logistic regression analyses were used. The null hypothesis states that there will be no relationship between the progression of time and the number of crypts infected with *L. intracellularis*.

To measure the correlation between cell infiltration and the level of infection of *L. intracellularis* the non-parametric Spearman rank correlation analysis was performed for all cell types examined ($p<0.05$ indicates significance). The null hypothesis states that there will be no correlation between the percentage of crypts infected with *L. intracellularis* and the number of infiltrating cells.

2.4 Polyacrylamide Gel Electrophoresis.

2.4.1 PAGE/Western Assay.

Plasma samples from mice were used for Western blot analysis. *L. intracellularis* proteins were separated by SDS-PAGE. Resolving gels for Tris-glycine SDS-PAGE had a final concentration of 12.5% acrylamide (2.52 ml of 40% solution acrylamide/bis-acrylamide 37.5:1, Sigma; 2.98ml Tris HCl, pH8.6; 80µl of a 10% SDS solution, 1.78 ml distilled water, 0.7ml of 1%w/v ammonium persulphate, and 10µl TEMED). The stacking gel had a final acrylamide concentration of 5% (0.25 ml of 40% acrylamide/bis-acrylamide, 0.24 ml Tris-HCl pH 6.8, 1.28 ml distilled water, 20µl of a 10% SDS solution, 4µl TEMED and 0.2 ml of a 1% w/v APS solution). Gels were run in Tris-glycine buffer containing SDS (25mM Tris, 0.1% SDS, 250mM glycine).

Samples of *L. intracellularis* were boiled for 3 minutes in sample buffer (200mM Tris-HCl pH7.0; 8% SDS; 10% glycerol; 20% v/v mercaptoethanol; 0.001% bromophenol blue) prior to electrophoresis through vertical slab gels using Bio-Rad Mini Protean II gel equipment. Gels were run at a constant 100 Volts until the dye front reached the bottom of the gel. A Sigma wide range marker was also separated on each gel.

2.4.2 Western Blotting.

Following SDS PAGE separation the proteins were transferred to a nitrocellulose membrane (0.2μm, Amersham) using a semi-dry electroblotter (Bio-Rad). Transfer was performed using a blotting buffer of 39mM glycine, 48mM Tris base, 0.037% SDS and 20% methanol, at pH8.3, for 25 minutes at a constant current of 2mA per square centimetre. Following blotting, non-specific sites on the membrane were blocked for 1 hour in a solution of 1% w/v fat-free dried milk (Marvel) in PBS.

This was followed with an hour-long incubation for both the primary and secondary antibodies (each stage being carried out on an orbital shaker and followed by a washing step with 0.05% PBST).

The primary antibodies consisted of diluted plasma samples (1/100) from *L. intracellularis* challenged and control mice. The HRP-conjugated secondary antibody (either goat anti-mouse HRP or rabbit anti-swine HRP, Dako) was used at a concentration of 1/1000, and was followed by the addition of DAB substrate (Vector), incorporating nickel solution.

The appropriate dilution for the primary plasma samples was ascertained by separating *L. intracellularis* preparations on a polyacrylamide gel and transferring this to nitrocellulose as detailed previously. Various dilutions of plasma samples from established positive and negative animals were used (as detailed previously) to determine the dilution that gave a strong positive signal, in conjunction with minimal background. The blots were analysed by Gel doc 2000 (Bio-Rad) using the programme Quantity One.

2.4.3 Protein assay.

The quantity of antigen used in the gel was determined by a protein assay (Lowry). 0.1ml of sample was incubated at 37°C for 30 minutes with 0.4ml NaOH. 0.1ml of this was then incubated with 1ml of solution C (see below) for 10 minutes at room temperature.

A further incubation of 30 minutes at room temperature followed the addition of 0.1ml solution D (1ml Folin and Ciocalteau's Phenol reagent + 1.36ml distilled water). The absorbance was monitored at 750nm, and compared to that of standards treated in the same way to calculate the protein concentration in the original sample. Standards were prepared by dilution of bovine serum albumin (100, 80, 60, 40, 20, 10, 5, 0 $\mu g.ml^{-1}$ BSA in distilled water).

(Solution A:1g Potassium-Sodium-Tartrate was dissolved in a minimum volume of distilled water, as was 0.5g $CuSO_4.5H_20$. 10ml 1M NaOH was added and the solution made up to 100 ml with distilled water. Solution B: 2% w/v $NaCO_3$ in 0.1M NaOH, prepared fresh. Solution C: 1ml solution A + 50 mls solution B).

3. RESULTS.

3.1 Preliminary assessment of an *in vivo* model infection system of wildtype and isogenic gamma interferon receptor knockout mice.

To establish an animal infection system for proliferative enteropathy 129/Sv/Ev wild-type and isogenic IFNγR$^{-/-}$ mice were challenged with a suspension of *L. intracellularis*, and then monitored for signs of disease and associated pathology. The study was conducted in two stages. Experiment One consisted of the inoculation of four mice in each challenge group at day 0, and the animals were then sampled over a 40-day period (with time points at 7, 14, 21, 28 and 40 days post inoculation). The exceptions to this were seen at the final time point (day 40) and the control groups (at day 21 only) which consisted of two animals in both the WT and IFNγR$^{-/-}$ groups (see Table 2.2). The second phase of the study (Experiment Two) was to continue the development of the animal infection system for proliferative enteropathy, and to validate the results obtained in the first challenge experiment using WT and IFNγR$^{-/-}$ mice in a more controlled study. Each group consisted of four mice inoculated with *L. intracellularis* and two control mice given SPG buffer at day 0, for both WT and isogenic knockout mice, which were then assessed at four time points over 35 days i.e. 14, 21, 28 and 35 days post-inoculation (see Table 2.2). The time points monitored were amended as a result of the first experiment in this pilot study (which illustrated that there was no evidence of infection at 7 days post-inoculation in either the WT or IFNγR$^{-/-}$ mice), therefore the study began at 14 days post-inoculation, and continued at weekly intervals until 35 days post-inoculation. Towards this final stage there was evidence that some of the infected IFNγR$^{-/-}$ mice demonstrated severe clinical signs of anorexia, in some cases leading to fatalities, and it was not considered necessary at this time to extend the study.

3.1.1 Weight loss and gross changes at necropsy.

Mice were monitored clinically throughout the period of the study, with the majority remaining healthy throughout (the parameters assessed ranged from mild symptoms such as a hunched posture or ruffled fur, to diarrhoea and lack of weight gain). In the second phase of the study a wild-type control and an IFNγR$^{-/-}$ control animal died within the first week from an unknown cause and were consequently omitted from the study. A pathologist examined the mice, and no signs of infection with *L. intracellularis* were demonstrated following necropsy and immunohistochemical staining of ileal and colonic regions. Three IFNγR$^{-/-}$ mice inoculated with *L. intracellularis* due to be euthanased on day 35 post challenge died prior to this point. One at 31 days post inoculation, which was not retrieved, and two were found dead at day 33 post challenge. Samples were taken from one of these mice only, the other being too autolytic to be of any diagnostic value. The two animals were markedly underweight (one weighing 13g, and the other 12g, in comparison to 22g for the IFNγR$^{-/-}$ control animal at day 35).

Since Experiments One and Two differed in various aspects of design (including inocula used) they will be dealt with separately in the following sections.

3.1.1A Experiment One.

In Experiment One the average weight of each group of mice was monitored before gavage, and then again before post-mortem. The trend illustrated that both the wild type and IFNγR$^{-/-}$ groups inoculated with *L. intracellularis* gained weight over the 40-day study at a similar rate (data not shown as individual weights were not assigned). No gross lesions or thickening of the mucosa were evident following necropsy.

3.1.1B Experiment Two.

Figure 3.1a illustrates gross changes evident in the colon of an IFNγR$^{-/-}$ mouse infected with *L. intracellularis*. It demonstrates an instance where a grossly thickened region of colon from an IFNγR$^{-/-}$ mouse inoculated with *L. intracellularis* was also haemorrhagic and displayed blood clots in the lumen of the gastrointestinal tract (a feature seen in acute cases in the pig and termed proliferative haemorrhagic enteropathy). Such haemorrhagic changes were not a feature of disease in the mouse and the gross changes noted in other infected IFNγR$^{-/-}$ mice were restricted to thickening of the colon and serosal corrugation. Such changes were seen at necropsy in the colon of 2 (of 4) IFNγR$^{-/-}$ animals inoculated with *L. intracellularis* at day 21; all IFNγR$^{-/-}$ challenged mice at day 28 (4); and 1 (of 1) remaining IFNγR$^{-/-}$ challenged mouse at day 35 (numbers were reduced at this final time point due to earlier fatalities, see 3.1.1). No gross changes were noted in any of the inoculated wild type mice, or either of the control groups (Figure 3.1b).

3.1.2 Histopathology.

Haematoxylin and eosin stained sections of paraffin-embedded ileum and colon were examined for evidence of lesions; in particular epithelial hyperplasia and the absence of secretory goblet cells in all inoculated and control animals. At no stage did any of the control mice (dosed with SPG buffer) demonstrate signs of intestinal pathology. A proportion of mice inoculated with *L. intracellularis* demonstrated increased crypt epithelial cell proliferation with an associated reduction in the number of goblet cells, lesions that were typical of PE (Lawson and Gebhart, 2000). (See later photographs for representative examples, Figure 3.4).

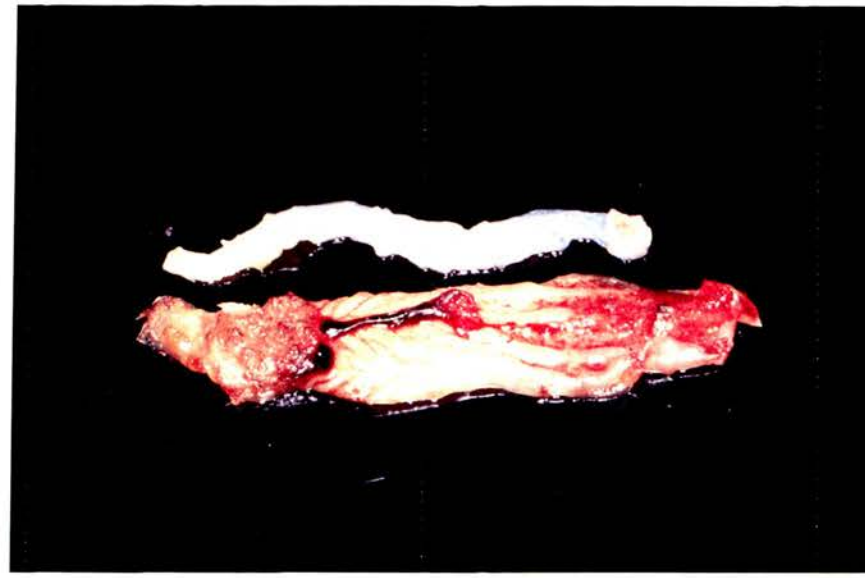


Figure 3.1a.

Figure 3.1b.

Figure 3.1. Experiment Two. Regions of colon from IFNγR$^{-/-}$ (a) and 129/Sv/Ev wildtype mice (b) at day 28 post-inoculation.
In both pictures the top region of the gastrointestinal tract is from the SPG dosed control mouse, and the lower section from an animal dosed with *L. intracellularis*.
Figure 3.1a demonstrates a grossly thickened region of the colon with blood clots in the lumen, markedly different from the control.
Figure 3.1b illustrates the close similarity between the two regions of colon.

3.1.3 Presence of *L. intracellularis* detected by immunohistochemical staining.

Immunohistochemical staining of the paraffin wax-embedded tissue sections of the ileum and colon used a polyclonal antibody specific for *L. intracellularis* (Lawson et al., 1985). Lesions of proliferative enteropathy clearly demonstrated *L. intracellularis* within the apical cytoplasm of hyperplastic immature enterocytes (and the bacteria demonstrated tropism for both the ileum and colon). The control mice remained consistently negative for the presence of *L. intracellularis.*

3.1.3A Experiment One.

At 7 days post-inoculation there were no signs of disease in either inoculated WT or IFNγR$^{-/-}$ mice whereas at day 14, 2 (of 4) WT mice demonstrated infection with *L. intracellularis*, with lesions in both the ileum and colon. Subsequently there was no evidence of infection in any of the inoculated WT mice. At day 14, 1 (of 4) IFNγR$^{-/-}$ inoculated mice were positive for *L. intracellularis* (with lesions in the colon only). At day 21, 3 (of 4) mice showed signs of proliferative enteropathy. Two of these had lesions in both the colon and ileum, and one in the ileum only. At day 28, 2 (of 3) mice demonstrated PE associated pathology, both infected animals had lesions exclusively in the colon. Within this group one mouse died prior to euthanasia, but was not retrieved for further analysis. At day 40, both remaining IFNγR$^{-/-}$ mice were positive for *L intracellularis*, one in both the colon and ileum, and one the colon only.

Figure 3.2 illustrates the percentage of crypts in the intestine that were infected with *L. intracellularis*, in both the ileum and colon, in all mice with lesions of proliferative enteropathy. This method of assessing the proportion of infected was used to calculate the degree of infection because of the obligate intracellular nature of the organism, and therefore the difficulty in calculating exact counts of the organism within an infected area. Results demonstrate that infection with *L. intracellularis* was only apparent at 14 days post inoculation in two of the wild-type mice, and was highest in the ileum in both cases of infection.

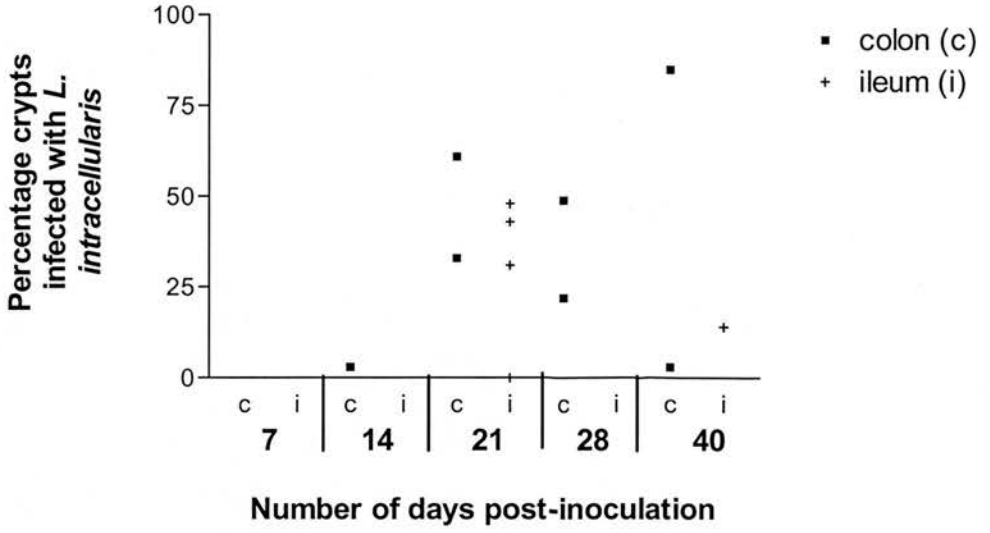


Figure 3.2a.

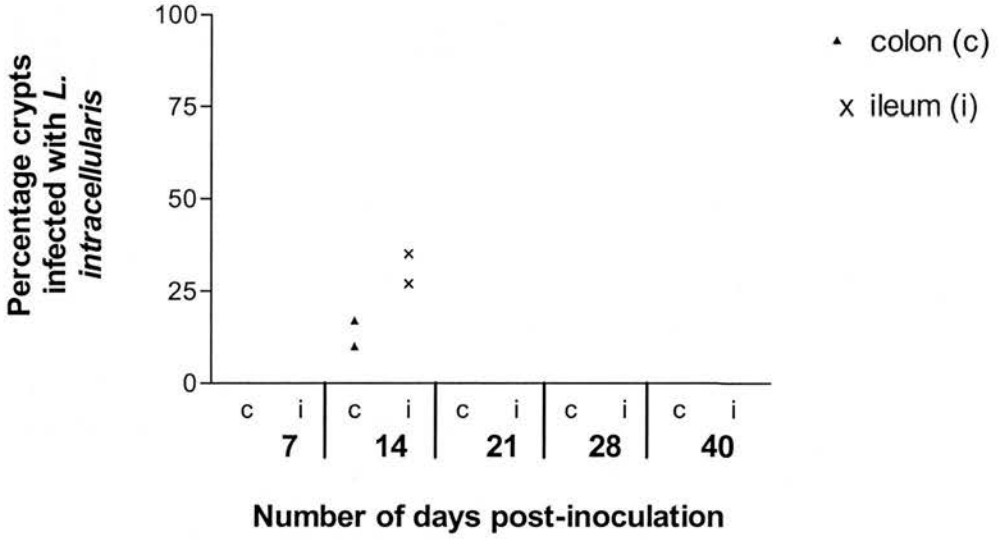


Figure 3.2b.

Figure 3.2. Experiment One. Percentage of crypts infected with *Lawsonia intracellularis* in all animals with lesions of proliferative enteropathy.
Figures 3.2a and 3.2b represent IFNγR$^{-/-}$ and WT mice respectively.
Each point represents the count for an individual animal (and was determined by calculating the average proportion of infected crypts in 10 random fields of view per section).

While the highest single incidence of infection was reached at day 40 in the colon of an IFNγR$^{-/-}$ mouse, the average peak was seen at day 21. Contrary to the wild type mice, a higher degree of crypts were infected with *L. intracellularis* in the colon, rather than the ileum in the IFNγR$^{-/-}$ animals. At day 28 there were no signs of infection in the ileum in either of the IFNγR$^{-/-}$ mice demonstrating colonic lesions, and at day 40 there was a low level of ileal infection (14%) in the mouse that presented with the highest proportion of infected crypts in the colon.

The immunohistochemical staining demonstrated that *Lawsonia intracellularis* was capable of initiating infection in both wild type and IFNγR$^{-/-}$ strains of 129/Sv/Ev mice. These results demonstrated that following a suitable challenge dose there was sufficient multiplication of the bacteria to establish disease, and that the pathology of this appeared to resemble that seen in the natural host. There was localisation of the bacteria within the apical cytoplasm of immature crypt cells in both the ileum and colon, as well as the exclusive hyperplasia of these infected enterocytes, and a reduction in the number of associated goblet cells. Two wild-type mice demonstrated infection at 14 days post-inoculation, with no further evidence of infection whereas infection in the IFNγR$^{-/-}$ mice remained at all time points from day 14 onwards.

3.1.3B Experiment Two.

Microscopic lesions consistent with proliferative enteritis were seen in at least three out of each group of four inoculated IFNγR$^{-/-}$ mice at all time points (i.e. proliferative crypts associated with the absence of goblet cells and the intracytoplasmic presence of *L. intracellularis*). Immunohistochemical staining demonstrated the consistent presence of *Lawsonia intracellularis* within the apical cytoplasm of enterocytes, in association with increased proliferation of the infected crypt cells. The percentage of infected crypts within the intestine (both ileum and colon) is illustrated in Figures 3.3, and representative photographs of infected WT and IFNγR$^{-/-}$ mice are illustrated in Figure 3.4. Control mice were consistently negative for the presence of *L. intracellularis.*

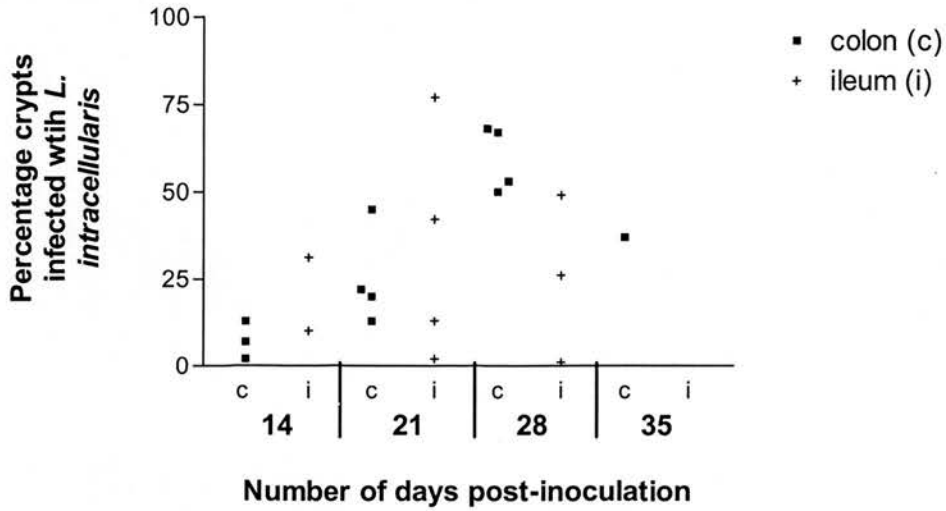


Figure 3.3a.

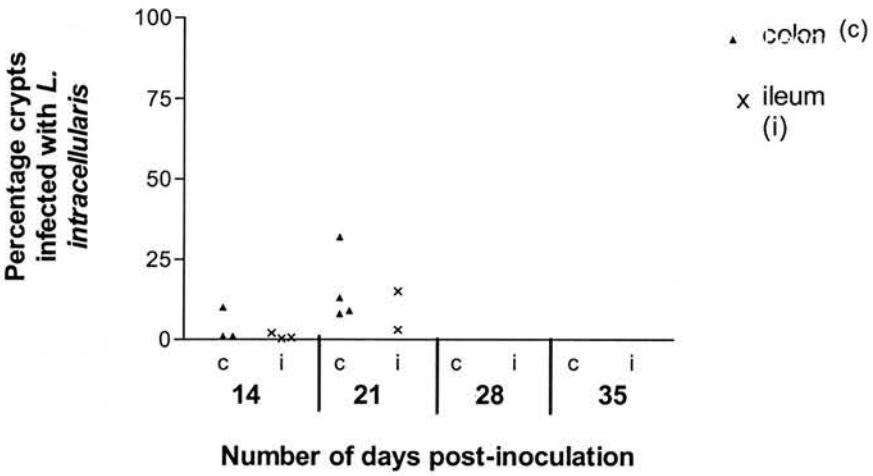


Figure 3.3b.

Figure 3.3. Experiment Two. Percentage of crypts infected with *L. intracellularis* in all animals with lesions of proliferative enteropathy.
Figures 3.3a and 3.3b represent IFNγR$^{-/-}$ and WT mice respectively.
Each point represents the count for an individual animal (and is determined by calculating the average proportion of infected crypts in 10 random fields of view per section of intestine).

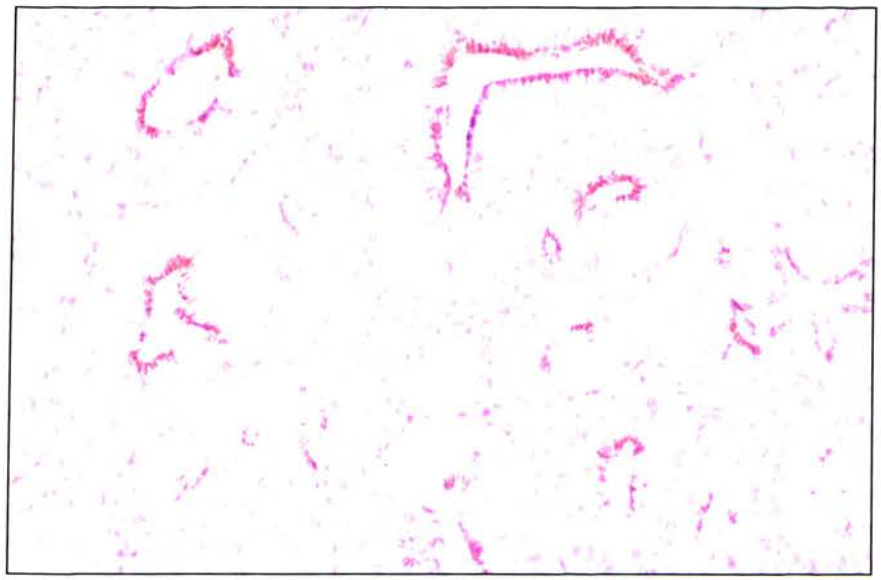


Figure 3.4a.

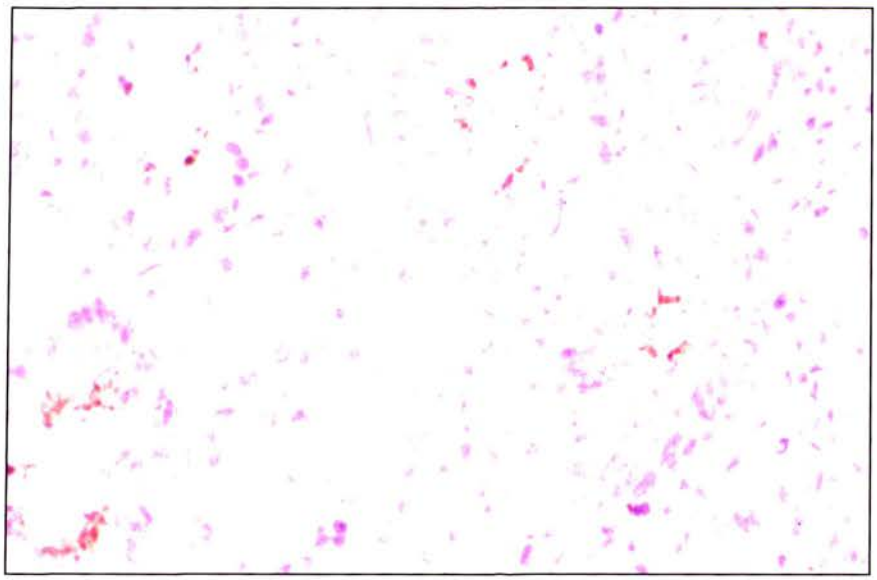


Figure 3.4b.

Figure 3.4 . Immunostained sections of colon from (a) IFNγR$^{-/-}$ (x250) and (b) WT mice (x400), 28 days post-inoculation with *L. intracellularis.*
A polyclonal antibody specific for *L. intracellularis* was used and demonstrates the presence of *L. intracellularis* within the apical cytoplasm of proliferating enterocytes. A greater degree of proliferation is seen in the IFNγR$^{-/-}$ mice than in the WT.

Wild type mice demonstrated lesions and the presence of *L. intracellularis* at day 14 in 3 (of 4) challenged mice, 2 in the colon and the ileum, and 1 in the ileum only. At day 21 all inoculated mice were positive for *L. intracellularis*, 2 in both the ileum and colon, and 2 in the colon only. At subsequent time points there was no evidence of infection or associated pathology. Mesenteric lymph nodes were also immunostained to look for the presence of *L. intracellularis,* but there were no bacteria evident at this site in either inoculated WT or IFNγR$^{-/-}$ mice.

L. intracellularis was present in 3 (of 4) inoculated IFNγR$^{-/-}$ mice at day 14, 2 in both the colon and the ileum, and 1 in the colon only. Four (of 4) IFNγR$^{-/-}$ mice had lesions of PE, and a demonstrable presence of *L. intracellularis* in both the colon and the ileum at day 21. At day 28, 4 (of 4) mice were infected with *L. intracellularis*, 3 in both the ileum and the colon. At day 35, the 1 remaining mouse had lesions of PE in both the ileum and colon. One of the mice that died by day 33 had a disseminated infection in the colon and ileum (Figure 3.5), with large numbers of organisms seen in aggregates in the lamina propria. Death was presumed to be due to infection with *L. intracellularis.* There was no evidence of *L intracellularis* in the liver, spleen or mesenteric lymph nodes (no data was available for the other two inoculated IFNγR$^{-/-}$ mice that died prior to day 35).

In some instances of proliferative enteropathy seen in IFNγR$^{-/-}$ infected mice bacteria were seen within the ileal villous tips and the mucosal folds of the colon surface epithelium (Figure 3.6), as well as the apical cytoplasm of immature enterocytes. This feature is not considered typical of the pig disease, and was seen only in infected IFNγR$^{-/-}$ mice and not in any wild-type mice with signs of proliferative enteropathy.
The results illustrated that disease can be reproduced in a proportion of wild-type and IFNγR$^{-/-}$ mice inoculated with pure cultures of *L. intracellularis*. The analogous disease pathology of PE seen in natural hosts, and in previous challenge studies, was also demonstrated.

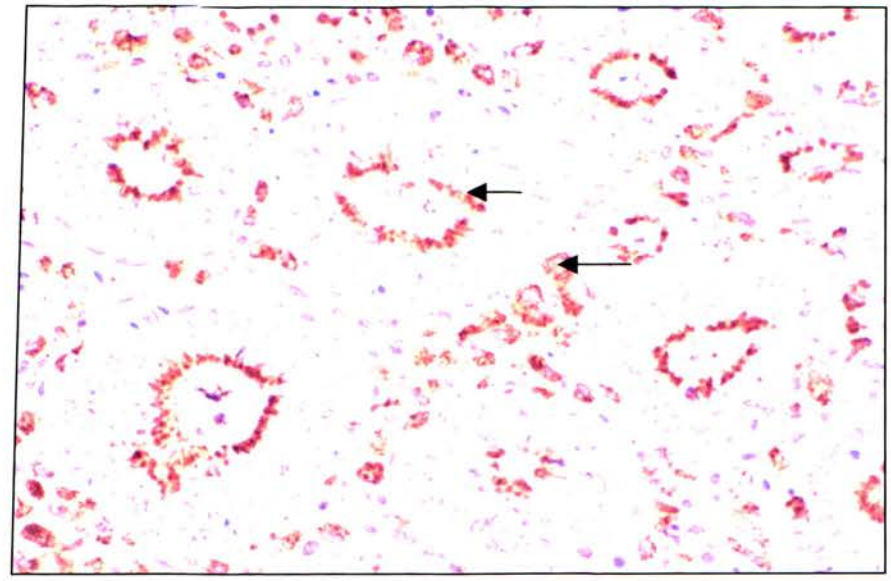


Figure 3.5. IFNγR$^{-/-}$ mouse that died 31 days post-inoculation. The arrows indicate *L. intracellularis* within the apical cytoplasm of proliferating enterocytes, as well as the presence of organisms within macrophages.

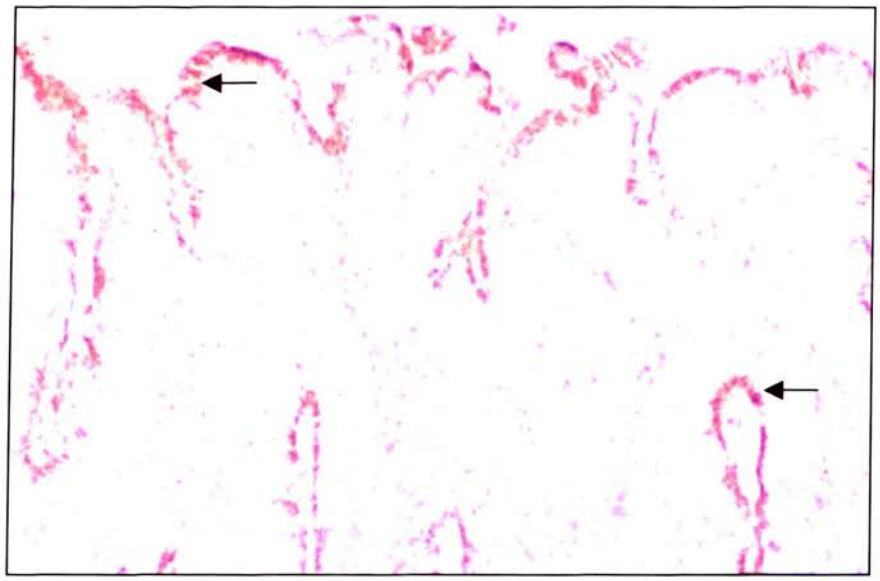


Figure 3.6. IFNγR$^{-/-}$ mouse challenged with *L. intracellularis* (21 days post-inoculation). The arrows illustrate *L. intracellularis*, stained using a specific polyclonal antibody. The organisms are seen both in the apical cytoplasm of enterocytes, and in the epithelium of the mucosal folds at the surface of the colon.

3.2 Experiment Three. Preliminary assessment of immune cell infiltration following inoculation with *L. intracellularis* in 129 Sv/Ev mice.

Following the establishment of a suitable infection system for *L. intracellularis* in 129 Sv/Ev mice the aim of the third challenge experiment was to begin investigation of the immune response that followed infection. 129/Sv/Ev and IFNγR$^{-/-}$ mice were inoculated with *L. intracellularis* in groups of 4 (and necropsies of each group carried out at 14, 21, 28 and 35 days post-inoculation, alongside a control group at each time point of 2 mice).

3.2.1 Weight loss and gross changes seen at necropsy.

The weight of each mouse was recorded prior to inoculation, and again prior to necropsy. The individual calculated weight differences are illustrated in Figure 3.7. The wild-type mice inoculated with *L. intracellularis* showed an initial weight loss, but then gained weight at days 28 and 35. With the exception of day 21, the WT control mice demonstrated a weight gain over the 35 days. The IFNγR$^{-/-}$ control mice showed an increasing weight gain over the 35 days, in comparison to the challenged IFNγR$^{-/-}$ mice which had an increase in average weight at day 14, but then a marked decrease over the remaining period.

Gross changes (such as mucosal thickening and serosal corrugation) were seen at necropsy in all IFNγR$^{-/-}$ mice inoculated with *L. intracellularis* at days 21, 28 and 35 post-inoculation (exclusively in the colon, with no apparent changes in the ileum). No marked gross changes were evident in control groups from either background. There was evidence of slight thickening of the WT colon, although to a lesser degree than that seen in infected IFNγR$^{-/-}$ mice. Again, no macroscopic changes were obvious in the ileum.

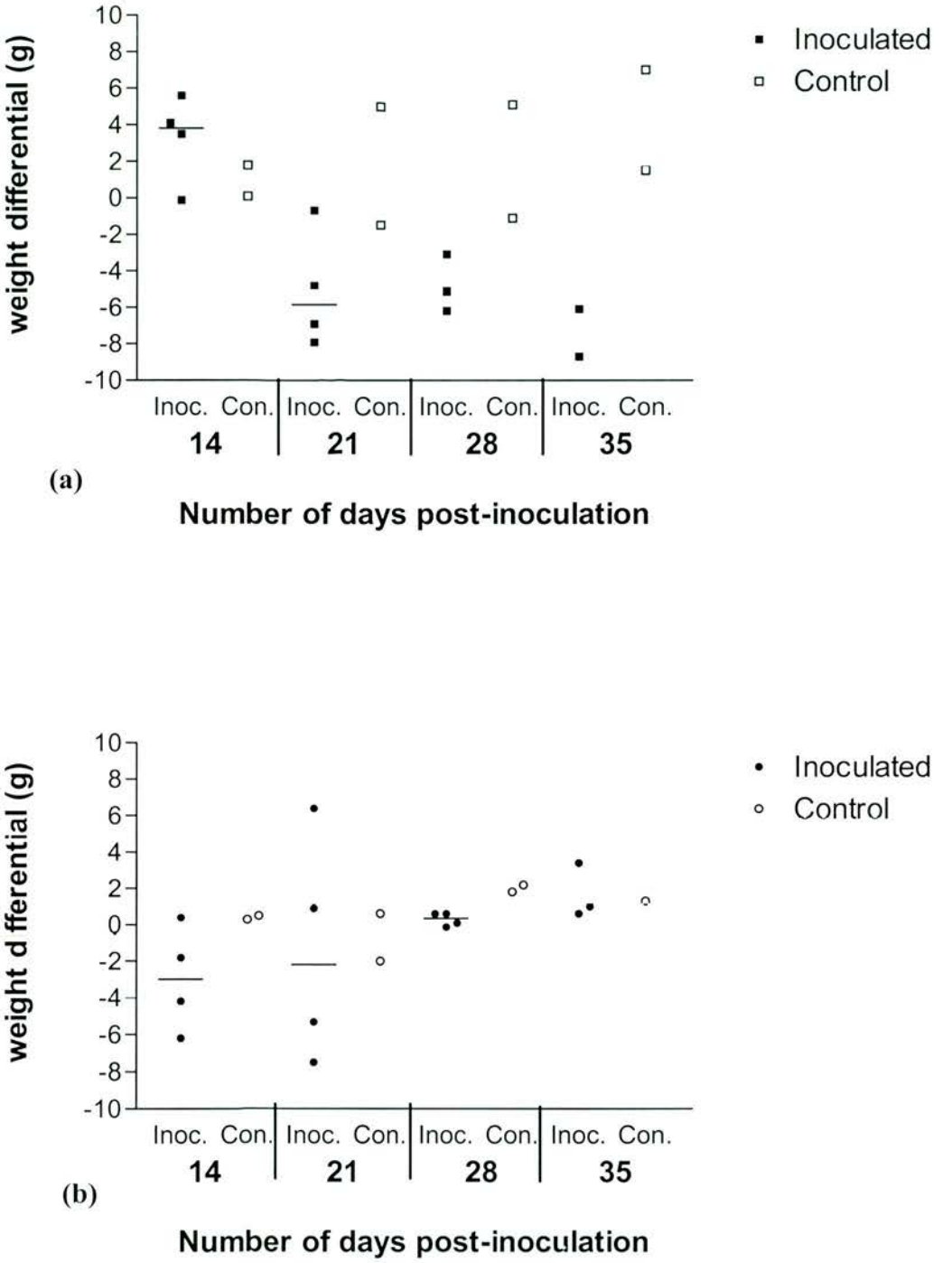


Figure 3.7. (a) IFNγR$^{-/-}$ and (b) WT mice.
Weight differential (g) in all mice between the day of inoculation with *L. intracellularis* and post mortem. Control animals were inoculated with SPG buffer only.
The scale represents either a positive or negative difference in overall weight gain.

3.2.2 Histopathology.

Haematoxylin and eosin (H+E) stained sections of colon were examined for lesions of PE, in particular crypt hyperplasia, and the presence of an inflammatory cell infiltrate as time progressed. Infected mice demonstrated increased crypt epithelial cell proliferation with associated reduction in the number of goblet cells, lesions typical of proliferative enteropathy. All control animals presented with a similar picture of well differentiated surface and crypt epithelial cells (Figure 3.8), in association with numerous goblet cells and scattered neutrophils and mononuclear cells within the lamina propria. Figures 3.9-3.15 illustrate typical changes following inoculation with *L. intracellularis*, and are summarised in Table 3.1.

3.2.3 *L. intracellularis* detected by immunohistochemical staining.

Colon and ileum samples were examined microscopically for evidence of infection with *L. intracellularis* (detected immunohistochemically). Wild-type mice displayed lesions of proliferative enteropathy at day 14 post-inoculation only, and all IFN$\gamma R^{-/-}$ mice inoculated with *L. intracellularis* developed PE. The localisation, and severity, of infection varied over time, and the results are summarised in Table 3.2 and Figure 3.16.

Strain	Days p.i.	Epithelium	Lamina propria inflammation	Figure no.
WT	14	Small focal areas of crypt cell hyperplasia in animals with PE Reduction in GC numbers	Mixed infiltration of M, PMN, L and PC	3.9
IFNγR$^{-/-}$	14	Hyperplasia and associated loss of GC	Mixed infiltration of M and L adjacent to proliferative crypts	3.10
WT	21		Chronic inflammation	3.11
IFNγR$^{-/-}$	21	Enterocyte hyperplasia and continued loss of GC	Ongoing inflammatory response incorporating PMN, M and L (with microabscess formation)	3.12
WT	28	No evidence of hyperplastic crypts, return of normal GC numbers	Minimal inflammatory response	3.13a
IFNγR$^{-/-}$	28	Hyperplastic crypts, loss of GC	Chronic active inflammation	3.14
WT	35	No evidence of hyperplastic crypts, return of normal GC numbers	Minimal inflammatory response with small focal areas of chronic inflammation	3.13b
IFNγR$^{-/-}$	35	Continued hyperplasia, with evidence of inflammatory debris in crypt lumens	Continued severe inflammatory reaction predominantly mononuclear (chronic)	3.15

Table 3.1. Summary of changes in the epithelium and lamina propria following inoculation with *L. intracellularis* in WT and IFNγR$^{-/-}$ mice.

GC-goblet cell. PMN-plymorphonuclear phagocyte. M-macrophage. PC-plasma cell. L-lymphocyte. IEL-intraepithelial lymphocyte

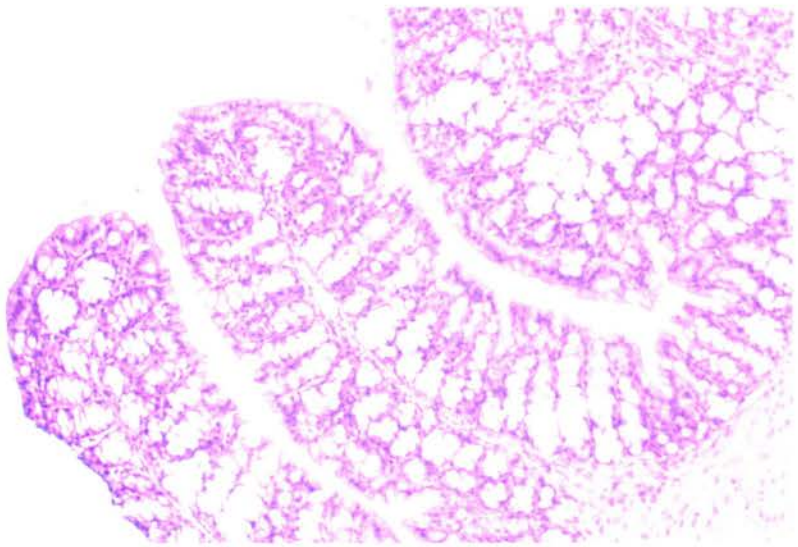


Figure 3.8a

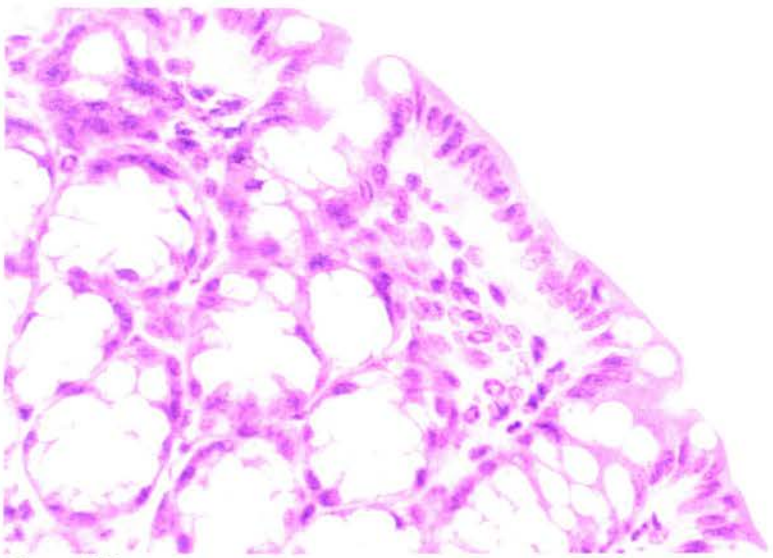


Figure 3.8b

Figure 3.8
Section of colon from wild type control mouse showing typical well differentiated surface and crypt epithelial cells with numerous goblet cells evident. Scattered neutrophils and mononuclear cells are evident in the lamina propria.
Haematoxylin and eosin. Figure 3.8a x100; Figure 3.8b x250

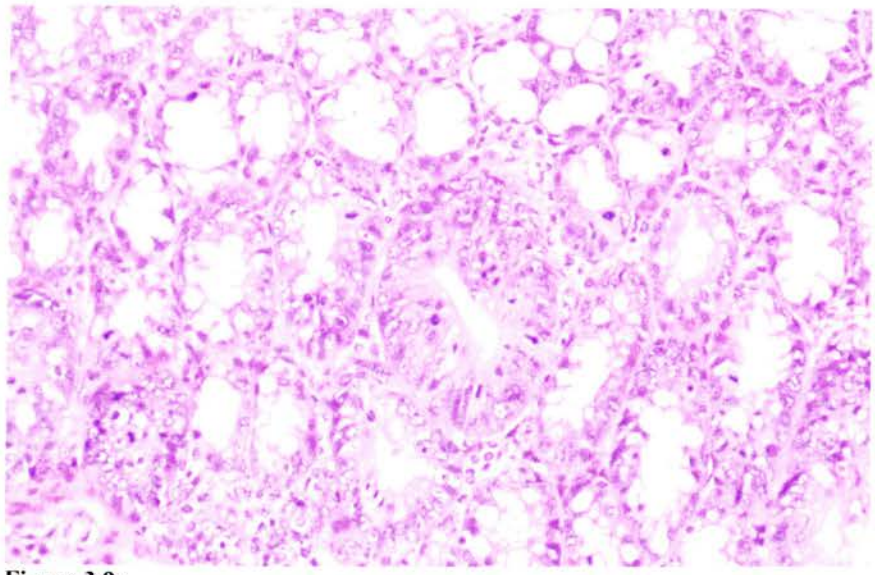


Figure 3.9a

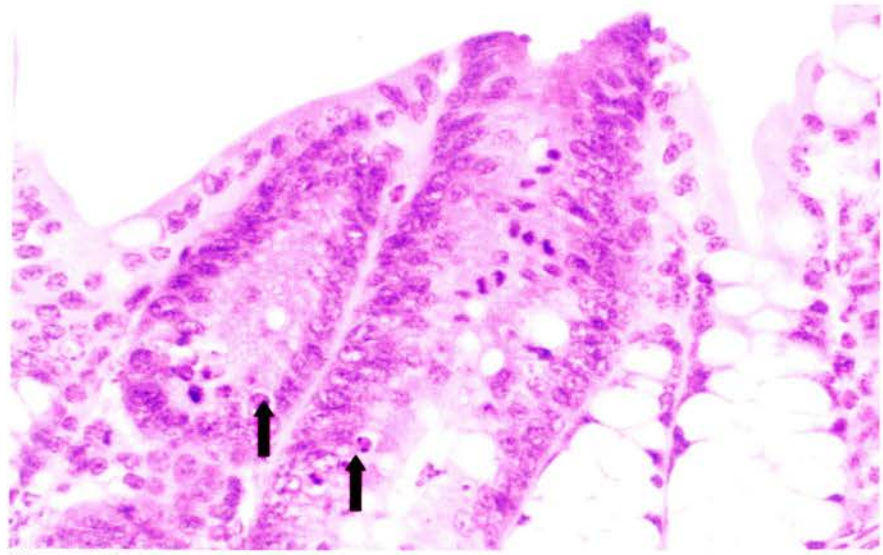


Figure 3.9b

Figure 3.9
Colon sections from wild type challenged mice at day 14.
Figure 3.9a shows a focal hyperplastic crypt with loss of goblet cells.
Figure 3.9b shows loss of goblet cell differentiation, increase in mitotic figures and apoptotic cells between epithelial cells (arrows).
H & E. Figure 3.9a x250; Figure 3.9b x400

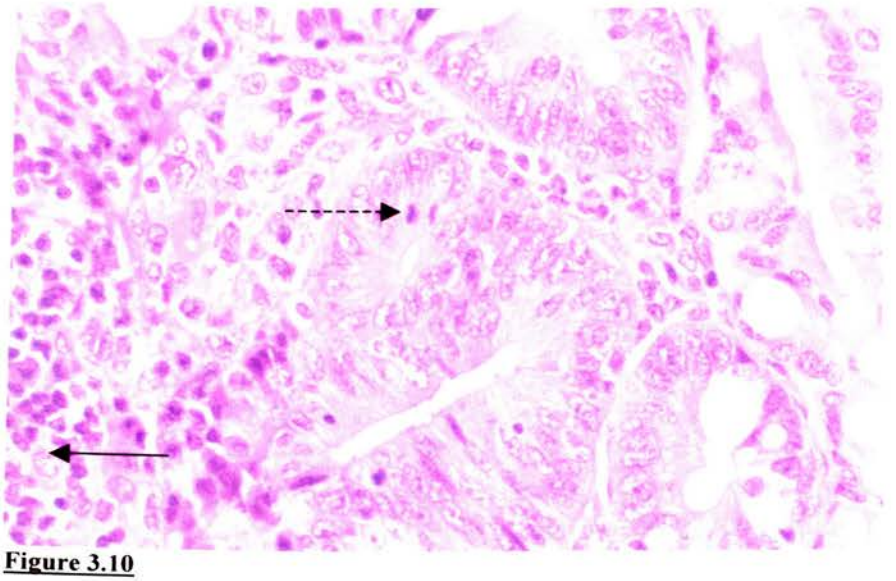


Figure 3.10

Figure 3.10
Section of colon from an IFNγR$^{-/-}$ mouse 14 days post inoculation with *L. intracellularis*. A hyperplastic crypt is evident with several mitotic figures (dashed arrow). There is an adjacent area of marked inflammation in the lamina propria including macrophages (solid arrow) and lymphocytes.
H & E x 250.

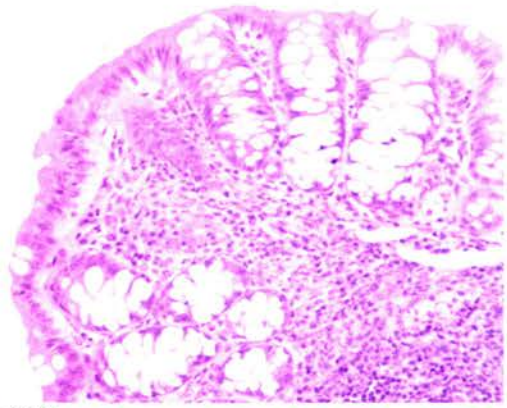


Figure 3.11a

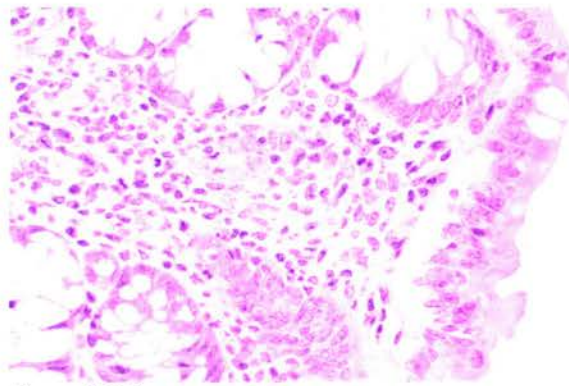


Figure 3.11b

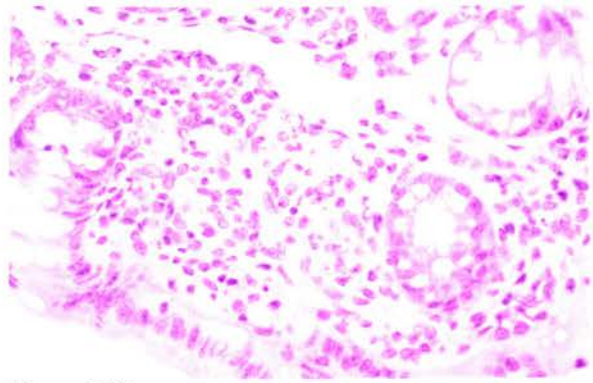


Figure 3.11c

Figure 3.11
Sections of colon from WT mice 21 days post inoculation with *L. intracellularis*, but negative for the presence of *L. intracellularis*.
All show marked but focal chronic active inflammation in the lamina propria.
H&E. Figure 3.11a x250; Figure 3.11b x400; Figure 3.11c x400

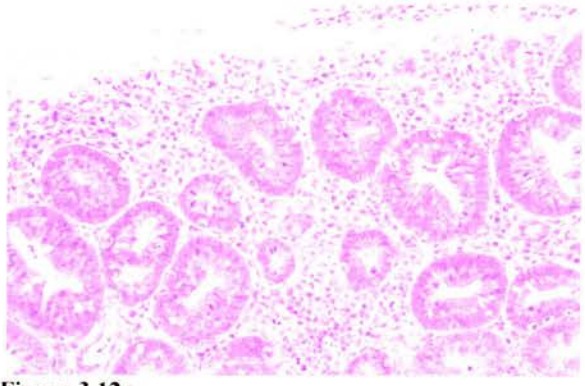


Figure 3.12a

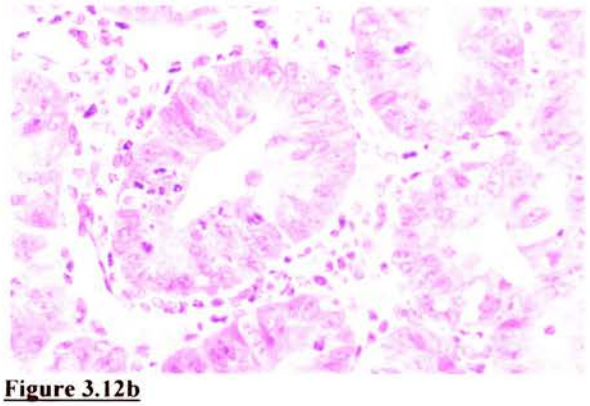


Figure 3.12b

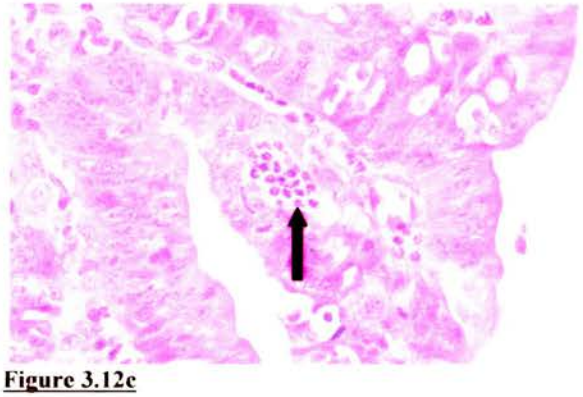


Figure 3.12c

Figure 3.12

Sections of colon from IFNγR$^{-/-}$ mouse 21 days post inoculation with *L. intracellularis*.
All show a marked inflammatory reaction in the lamina propria with the majority of crypts being hyperplastic. In addition, Figure 3.12b shows neutrophils trafficking between the hyperplastic crypt epithelial cells and Figure 3.12c shows an intraepithelial microabscess (arrow).
H&E. Figure 3.12a x250; Figure 3.12b x 400; Figure 3.12c x 400.

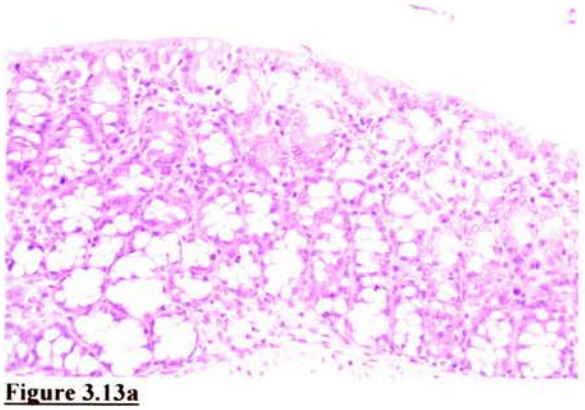


Figure 3.13a

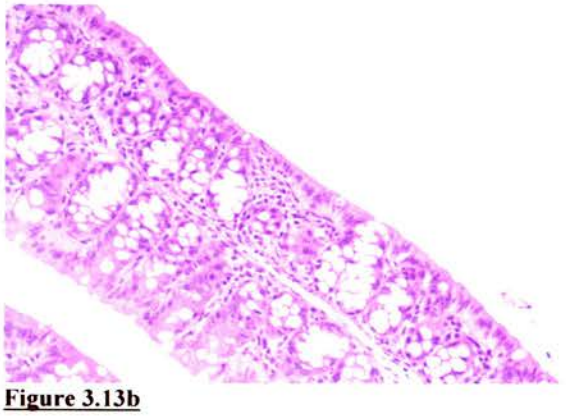


Figure 3.13b

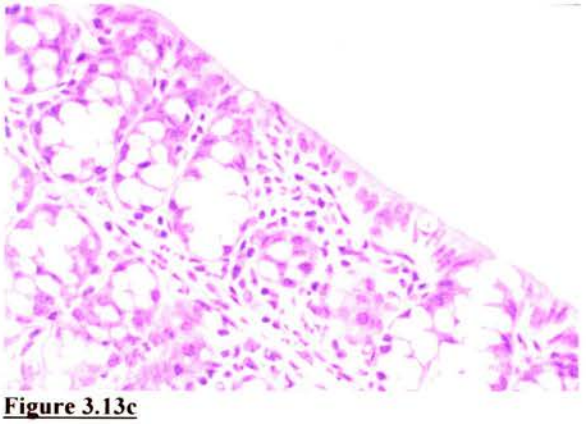


Figure 3.13c

Figure 3.13
Sections of colon from wild type mice inoculated with *L. intracellularis* but not infected.
Figure 3.13a: day 28 post inoculation showing very mild lamina propria inflammation.
Figure 3.13b: day 35 post inoculation showing normal structure with small focus of chronic inflammation
Figure 3.13c: higher power of 3.13b
H&E. Figure 3.13a x250; Figure 3.13b x250; Figure 3.13c x 400

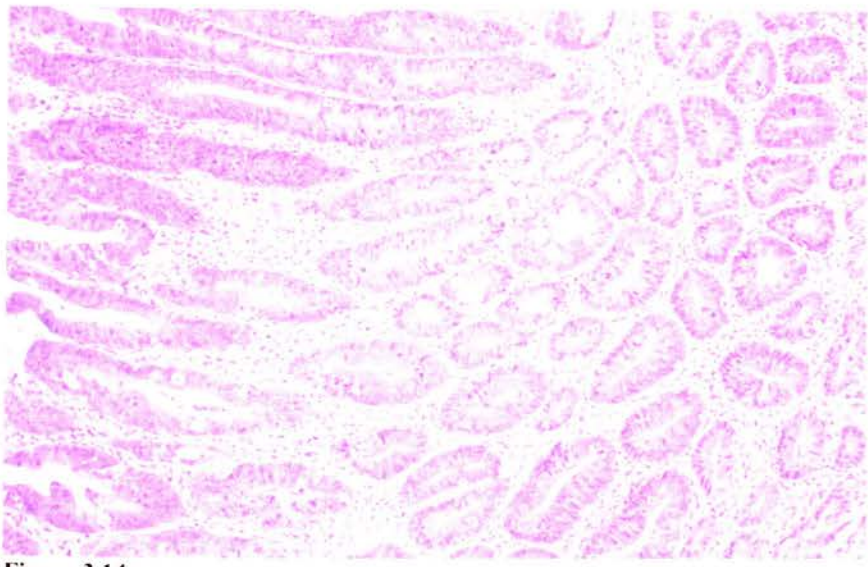


Figure 3.14a

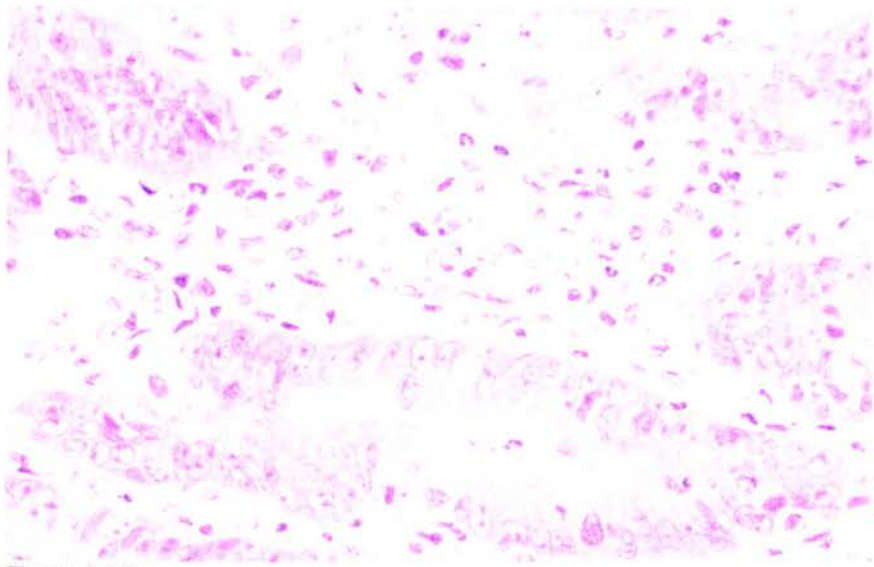


Figure 3.14b

Figure 3.14
Sections of colon from IFNγR$^{-/-}$ mice 28 days post inoculation with *L. intracellularis*.
Figure 3.14a shows variable crypt hyperplasia with scattered goblet cells. There is a marked inflammatory reaction in the lamina propria.
Figure 3.14b shows the mixed nature of the inflammatory reaction (macrophages, lymphocytes and neutrophils).
H&E. Figure 3.14a x100; Figure 3.14b x400

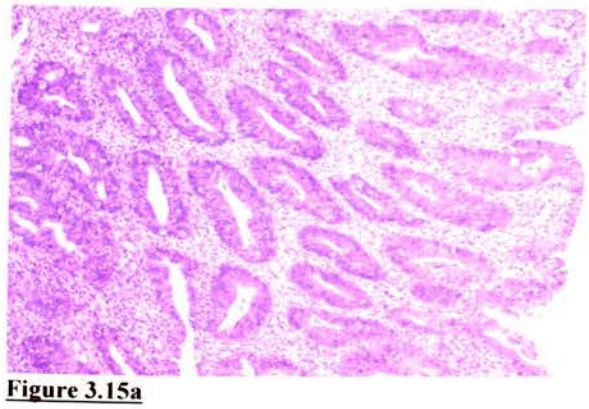


Figure 3.15a

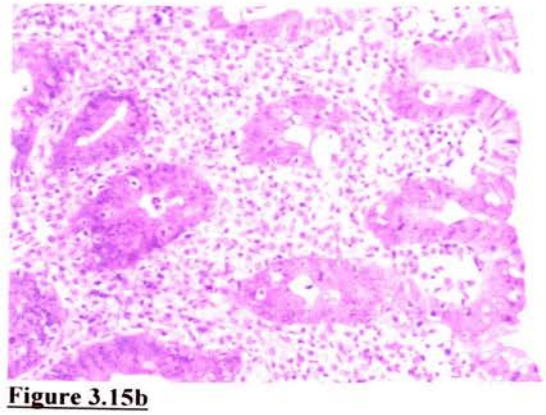


Figure 3.15b

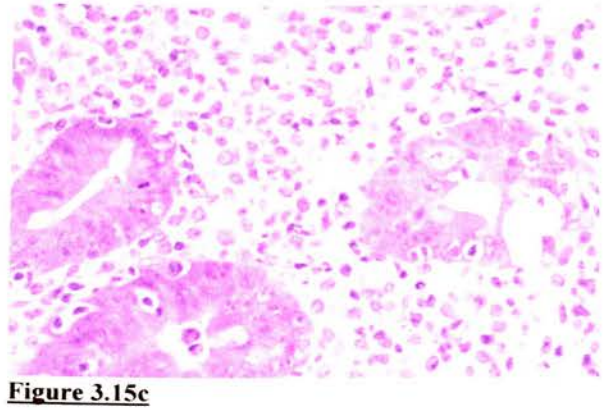


Figure 3.15c

Figure 3.15

Sections of colon from IFNγR$^{-/-}$ mice 35 days post inoculation with *L. intracellularis*.
Figure 3.15a and 3.15b show marked epithelial hyperplasia and lamina propria inflammation. Figure 3.15c is a higher power showing the mixed nature of the inflammatory response and inflammatory debris in crypt lumens
H&E. Figure 3.15a x100; Figure 3.15b x250; Figure 3.15c x400.

Strain	Days p.i.	Infection	Region	Comments
WT	14	3/4	2 in colon only and 1 in the ileum only	
IFNγR$^{-/-}$	14	4/4	2 in both ileum and colon,1 ileum only and 1 colon only	
WT	21	0/4		
IFNγR$^{-/-}$	21	4/4	2 in both ileum and colon, 2 in colon only	
WT	28	0/4		
IFNγR$^{-/-}$	28	5/5	3 in both ileum and colon, 2 in colon only	All mice demonstrated signs of illness including diarrhoea, failure to thrive, ruffled fur, hunched posture and reduced activity
WT	35	0/3		
IFNγR$^{-/-}$	35	2/2	Both in the colon only	

Table 3.2. Summary of the numbers of mice in each group infected with *L. intracellularis*, and whether the organism was detected in the ileum or colon. At day 28 there was a group size of 6 following the incorporation of 2 mice previously in the group for day 35 following the death of one, and the euthanasia of the other due to illness (one was too autolytic at post-mortem for further examination).

Figure 3.16 illustrates the percentage of crypts infected with *L. intracellularis* in each infected animal (over the four time points). In WT mice infection was only detected at day 14 and was comparatively lower to that detected in IFNγR$^{-/-}$ mice. The data illustrates that in IFNγR$^{-/-}$ diseased mice the average level of infection in both the ileum and colon increased from day 14, to reach a peak at day 28, and then declined to day 35. It also illustrated, as in the WT mice, that the percentage of infected crypts was higher in the colon than in the ileum (at all time points).

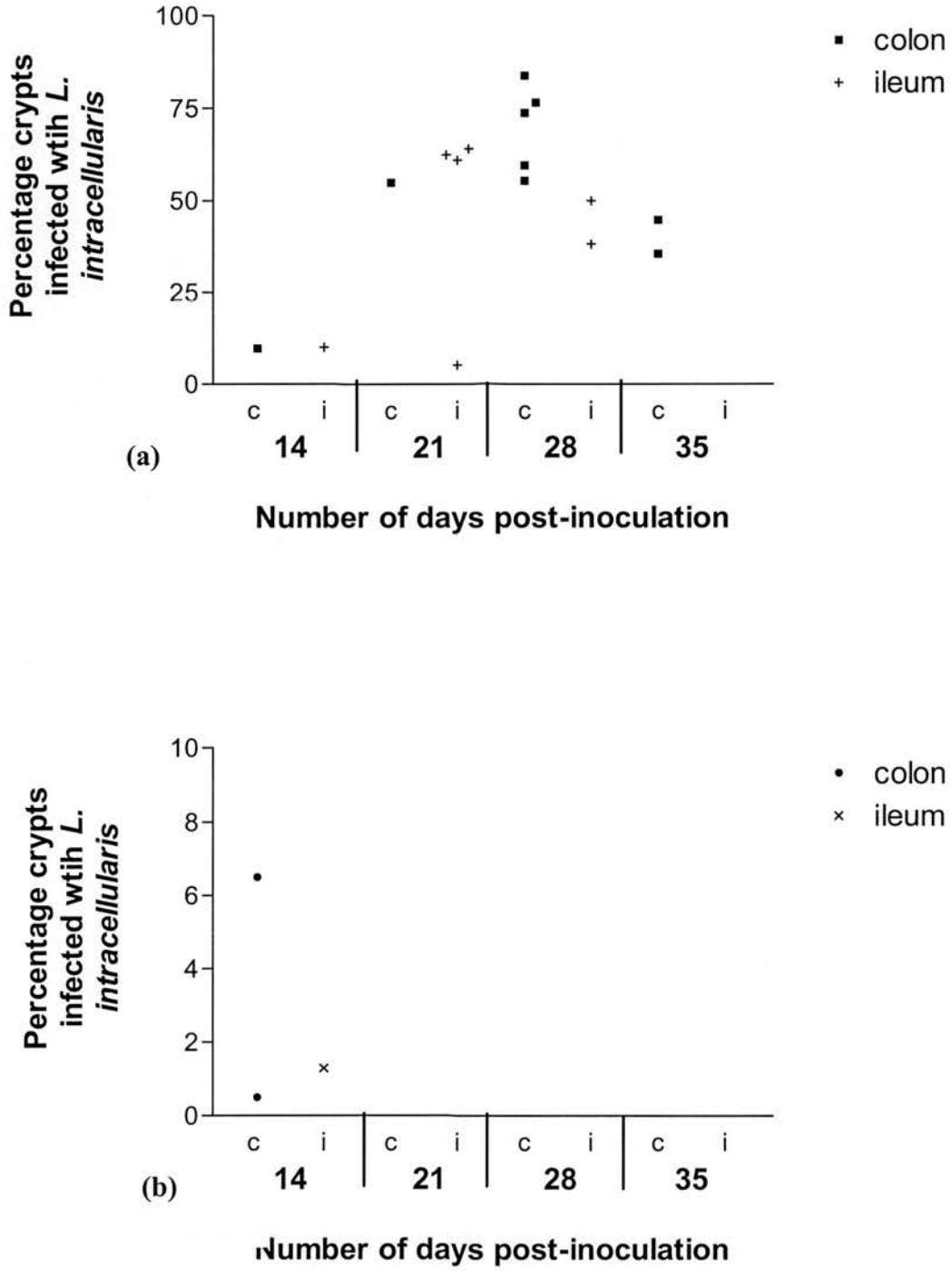


Figure 3.16. (a) IFNγR$^{-/-}$ and (b) WT mice.
Percentage of crypts infected with *L. intracellularis* in all animals with lesions of proliferative enteropathy following inoculation with *L. intracellularis*. Control mice were inoculated with SPG only.
Each value represents the count for an individual animal (and is determined by calculating the average proportion of infected crypts in 10 random fields of view per section of intestine).

Although not a normal feature of *L. intracellularis* infection, the presence of organism in mesenteric lymph nodes, Peyer's patches and lymphoid follicles of the colon was assessed immunohistochemically. There was no evidence of the organism in these sites in control, inoculated, or diseased animals. See Figure 3.17.

Figure 3.17a.

Figure 3.17b.

Figure 3.17c.

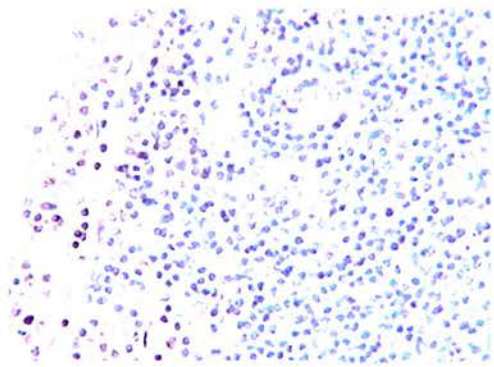


Figure 3.17d.

Figure 3.17e.

Figure 3.17f.

Figure 3.17. Typical illustrative examples of immunohistochemical staining for *L. intracellularis* in (i) mesenteric lymph node, (ii) Peyer's patch, and (iii) colonic lymphoid follicle which proved consistently negative.
Figure 3.17 a and b illustrate sections of MLN (x40 and x400 respectively).
Figure 3.17 c (x100) and d (x400) illustrate sections of ileal Peyer's patch.
Figure 3.17 e (x100) and f (x400) illustrate sections of colonic lymphoid follicle.

3.2.4 Phenotyping studies.

Following immunohistochemical staining of snap-frozen sections of colon (as detailed in section 2.3.2.4) using monoclonal antibodies specific for $CD4^+$ and $CD8^+$ T cells, counts of the number of positively stained cells for each individual animal were assessed. This was accomplished by averaging the number of positively stained cells over 10 random fields at x400 magnification (Figures 3.18 and 3.19). The CD8 counts for the wild-type mice inoculated with *L. intracellularis* remained similar to those obtained for the control sections over the 35 days, with the exception of 2 individual animals at day 21 which appeared elevated. In contrast, the $IFN\gamma R^{-/-}$ mice inoculated with *L. intracellularis* had an apparent elevated CD8 count for days 28 and 35 post challenge in comparison to the controls. Those at days 14 and 21 days post challenge were not markedly increased from the control (although due to the small group sizes statistical significance could not be unequivocally determined). $CD4^+$ T cell counts appeared elevated for both challenged WT and $IFN\gamma R^{-/-}$ mice at all time points looked at, in comparison to the controls. For WT mice a peak was reached at day 21 post-inoculation, and then the counts began to decrease (as infection with *L. intracellularis* also diminished). For challenged $IFN\gamma R^{-/-}$ mice the value for $CD4^+$ stained cells remained fairly constant over the time studied (but was generally lower than that seen for WT mice inoculated with *L. intracellularis*, irrespective of the presence of disease). One $IFN\gamma R^{-/-}$ mouse at day 21, and the other at day 35 had notably elevated CD4 counts in comparison to the other mice. For the mouse at day 35 this was also associated with a lower level of *L. intracellularis* infection than the other remaining infected $IFN\gamma R^{-/-}$ at this time point. These demonstrated that the two animals presented with a marked difference in the presence of $CD4^+$ cells, while both have a similar level of CD8 intraepithelial lymphocytes present. Both mice had an elevated cell count in comparison to the illustrated control.

The data was incomplete for some CD4 and CD8 counts due to the irretrievable loss of samples of frozen tissue before all immunohistochemical staining had been completed.

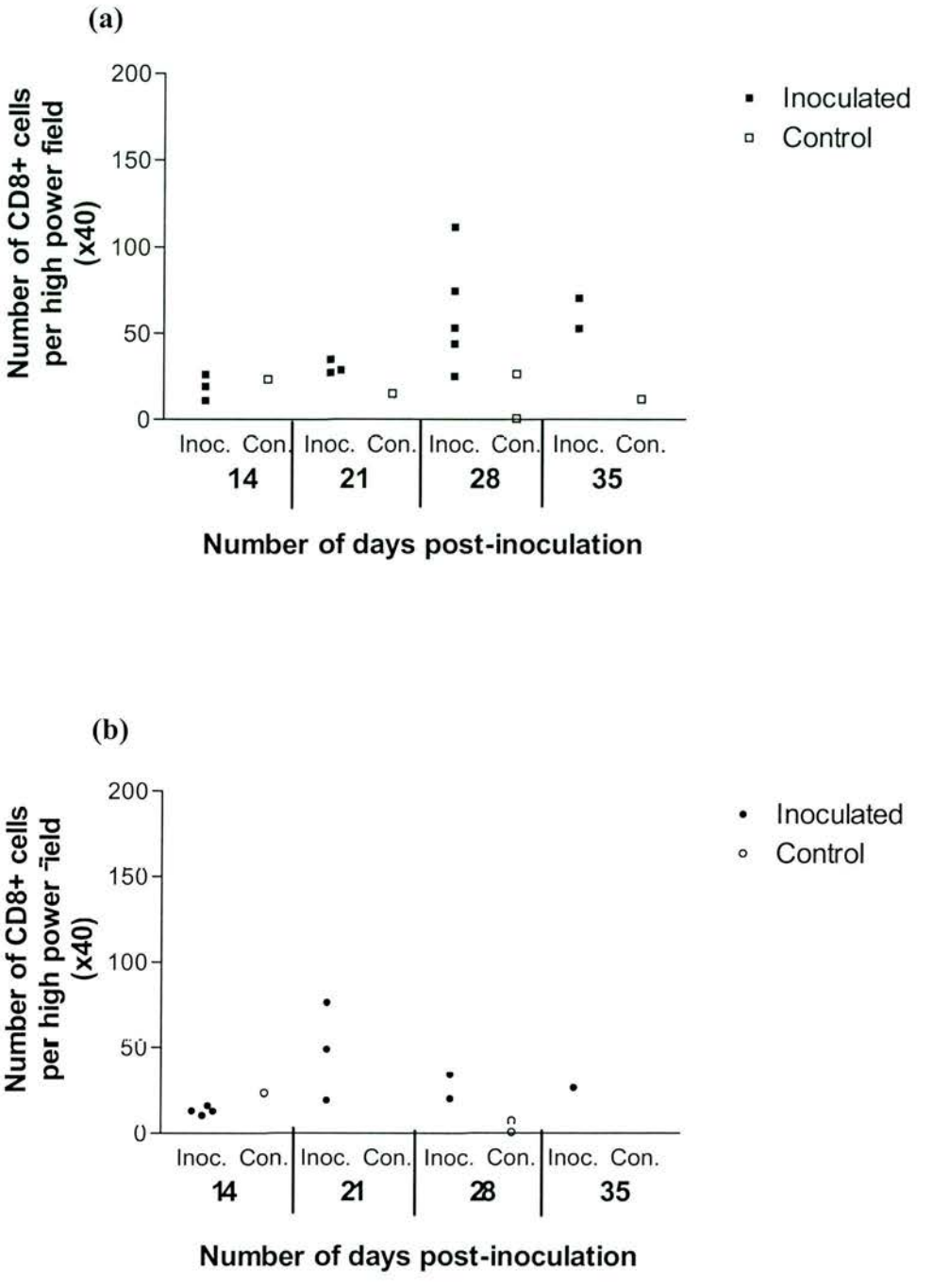


Figure 3.18 . $CD8^{+}$ cell infiltration in the colon, for inoculated and control groups of mice from both $IFN\gamma R^{-/-}$ (a), and WT (b) backgrounds.
Results were calculated as the average number of positively stained cells per field (x400), for 10 random fields in each section of tissue.
Individual data points for each animal are shown.

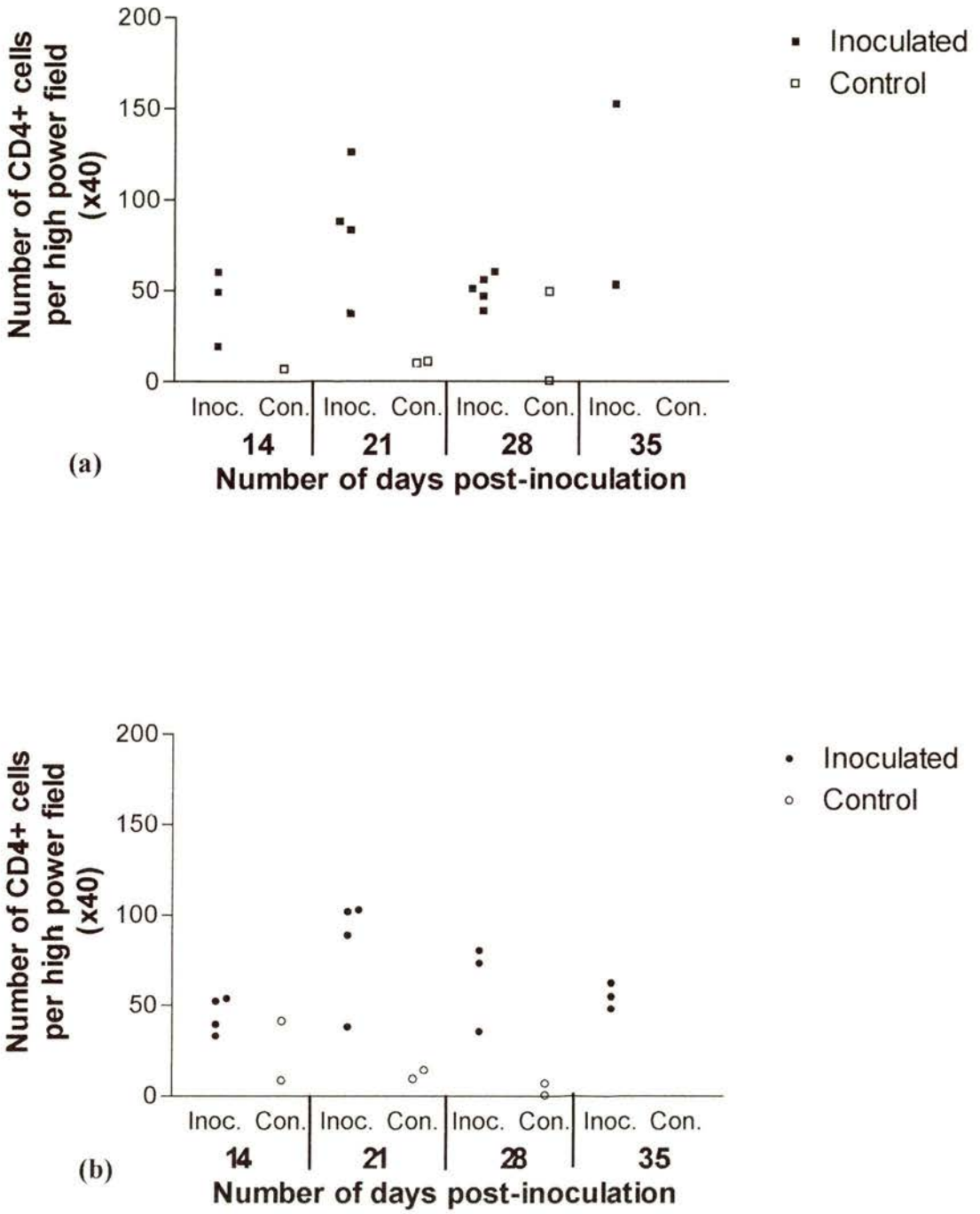


Figure 3.19. $CD4^+$ cell infiltration in the colon of inoculated and control groups of mice from both $IFN\gamma R^{-/-}$ (a), and WT (b) backgrounds.
Results were calculated as the average number of cells per field (x400), for 10 random fields in each section of tissue.
Individual data points for each animal are shown.

Therefore analysis of the results is further hampered by the small numbers in the study, and in some cases the absence of comparative control values.

3.2.5 Electron microscopy.

Colonic tissue was taken at necropsy and processed for transmission electron microscopy. Figure 3.20 illustrates that epithelial cells from a control IFNγR$^{-/-}$ animal (inoculated with buffer only) do not demonstrate any cytoplasmic bacterial organisms, and that they appear to have normal microvilli. Figure 3.14b demonstrates organisms consistent with *L. intracellularis* residing freely (non-membrane bound) in the cytoplasm of cells found at the tips of the mucosal folds of the colonic epithelium of from inoculated IFNγR$^{-/-}$ mice. There was also an apparent difference in the structure of the cell's microvilli, with potentially stunted growth seen in the infected cell.

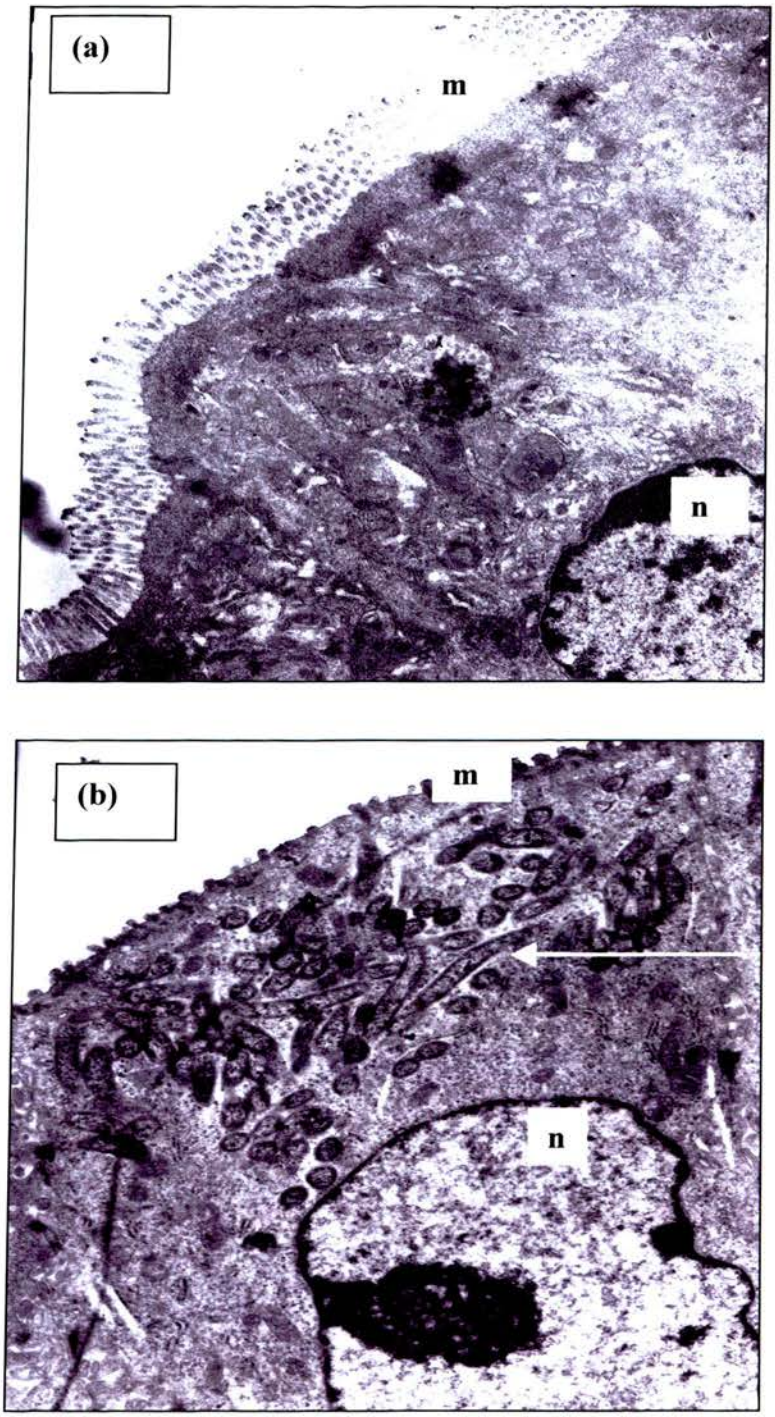


Figure 3.20. Electron micrograph from two IFNγR$^{-/-}$ mice at 35 days post-inoculation. X10000

Figure 3.20a. Control animal (inoculated with buffer only). The cell nucleus and microvilli can be clearly seen.
Figure 3.20b. Mouse inoculated with *L. intracellularis*, and determined positive for proliferative enteropathy immunohistochemically. The cell nucleus (n) can be clearly seen in both infected and control animals. Microvilli (m) show stunted appearance in infected cells (b). Intracellular, non-membrane bound bacterial organisms (arrow) can be clearly seen in the apical cytoplasm of cells from animals challenged with *L. intracellularis*.

3.3 Experiment Four.

The results from section 3.2.4 were suggestive of effects on cellular infiltration but were insufficiently discriminatory due to small sample size to obtain statistically significant data. The aim of the fourth challenge study was therefore to generate data that permitted a more comprehensive statistical analysis of the immune response following inoculation with *L. intracellularis*. Group sizes were increased for improved statistical power in the analysis of results. Consequently each of the time points assessed consisted of an inoculated group of 10 mice, and a control group of 5 animals (for both wild-type and IFN$\gamma R^{-/-}$ mice at 14, 21 and 28 days post-inoculation).

3.3.1 Weight loss and gross changes seen at necropsy.

Macroscopic alterations within the ileum and colon were assessed at post-mortem, and consisted of gross changes and thickening of the colon and ileum in a proportion of animals inoculated with *L. intracellularis*. Two inoculated WT mice necropsied at day 14 were the only animals that presented with thickened colons (they were later shown to possess lesions of proliferative enteropathy following immunohistochemical staining, see 3.3.3). Several animals at 21 days post-inoculation had evidence of blood in the duodenum, and although samples were taken there was no evidence of lesions of proliferative enteropathy. A proportion of inoculated IFN$\gamma R^{-/-}$ mice demonstrated gross pathological changes at all time points post-inoculation. At days 14 and 21 there were 3 of 10 animals in each group that presented with gross thickening of the colon (those at day 21 also demonstrated changes in the ileum). All animals that survived the full 28 days of the challenge study presented with gross thickening of the colon (with two also showing changes in the ileum). Several animals also had evidence of bloody faeces, and in two cases blood clots in the lumen.

The weight of each mouse was measured prior to gavage with *L. intracellularis*, and then again at post-mortem. Figure 3.21 illustrates the individual weight differential for each mouse between inoculation and necropsy.

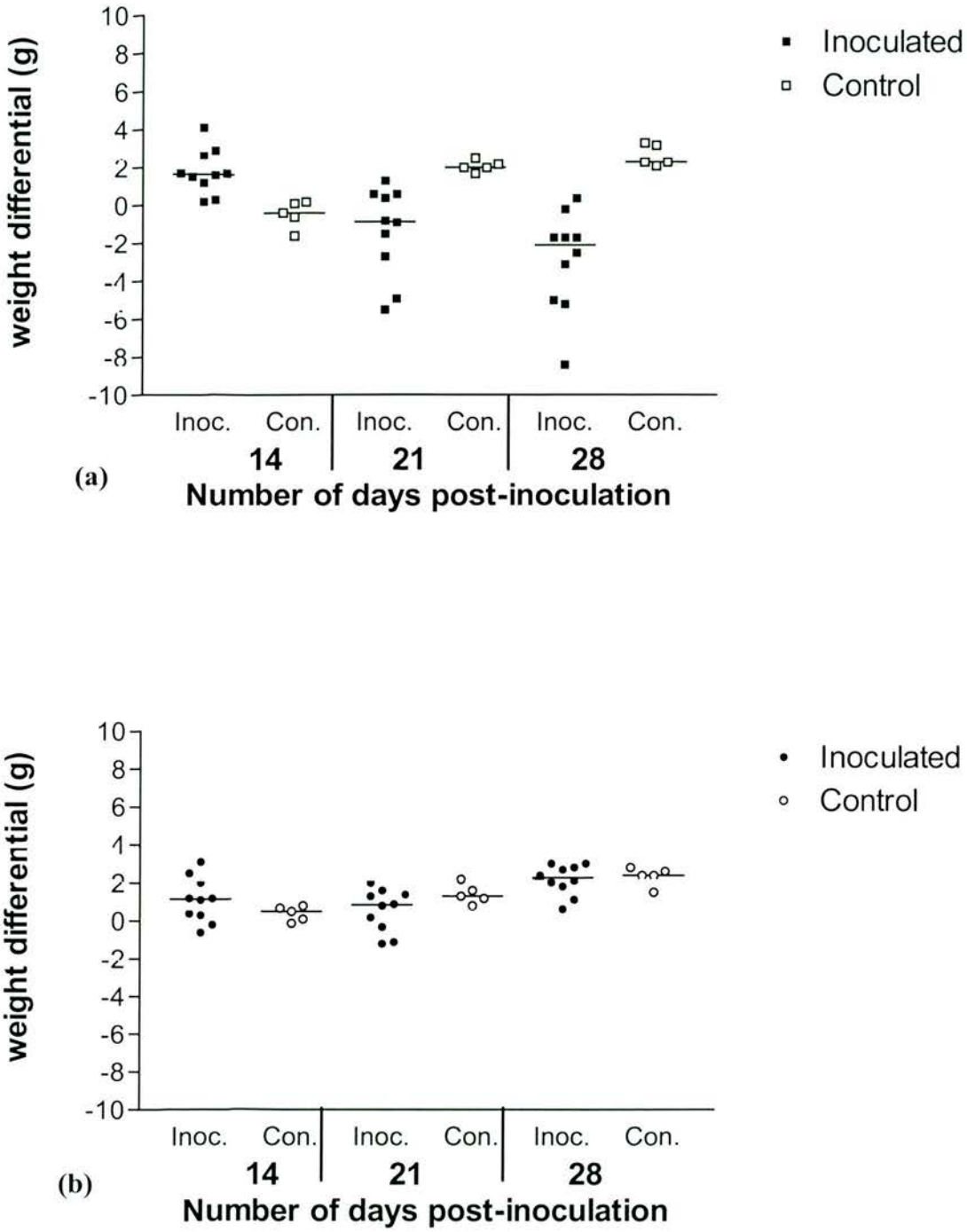


Figure 3.21. (a) IFNγR$^{-/-}$ and (b) WT mice.
Weight differential (g) in all mice between the day of inoculation with *L. intracellularis* and post-mortem. Control animals were inoculated with SPG buffer only. The scale represents either a positive or negative difference in the final weight.

The data illustrates that there was a gradual weight gain in IFNγR$^{-/-}$ control mice, but that IFNγR$^{-/-}$ mice inoculated with *L. intracellularis* (all of which developed lesions of PE) demonstrated varying degrees of weight loss. Analysis of these changes demonstrated a significant difference between the two groups for both the intercept ($p<0.001$) and the slope ($p<0.001$). In other words significantly more weight was lost on average by each mouse as time, and consequently disease, progressed. Figure 3.21b demonstrates that WT control and inoculated mice achieved a similar and gradual increase in weight over the study. These changes were assessed using the GLM and no significant differences between the two groups were ascertained (intercept, $p=0.311$ and slope, $p=0.251$).

3.3.2 Histopathology.

Haematoxylin and eosin stained sections of paraffin-embedded ileum and colon were examined for evidence of lesions, in particular epithelial hyperplasia. A proportion of inoculated mice demonstrated increased crypt epithelial cell proliferation with an associated reduction in the number of goblet cells; these lesions were typical of proliferative enteropathy.

Examination of haematoxylin and eosin stained sections included analysis of inflammation in the lamina propria as time, and disease, progressed (Appendix II), and changes in the epithelium were also noted. It was evident that WT mice inoculated with *L. intracellularis* displayed some focal hyperplasia with associated goblet cell loss, and that this was evident only at day 14 post-inoculation. At days 21 and 28 there were no notable alterations in the epithelium. In contrast a mild to moderate increase of inflammatory cells (including lymphocytes, polymorphonuclear phagocytes, plasma cells and macrophages) was seen in the majority of mice at days 14 and 21, reducing to only 3 (of 10) at the final time point. The changes noted in inoculated IFNγR$^{-/-}$ mice were notably more severe. Evident changes in the epithelium ranged from small focal areas to marked diffuse crypt hyperplasia, and was consistently associated with goblet cell loss. Such changes could be detected in all inoculated IFNγR$^{-/-}$ mice (with the exception of one at day 28, which was found dead and was too autolytic to be of diagnostic value). Inflammatory cell infiltration into the lamina propria was also apparent in all animals.

At day 14 this consisted primarily of a mild increase in lymphocytes, PMN, plasma cells and macrophages, extending to a moderate/marked increase by days 21 and 28 post-inoculation.

The average number of enterocytes per crypt is presented in Figure 3.22 for inoculated and control mice. Enterocyte counts for each animal were averaged from counts of crypts from ten random fields from each section. GLM analysis performed on logged data for IFNγR$^{-/-}$ mice failed to demonstrate any statistical significance in either the intercept ($p=0.357$) or the slope ($p=0.308$) between the inoculated and control groups of mice. However the number of enterocytes per crypt was significantly greater in infected IFNγR$^{-/-}$ mice in comparison to control animals following analysis using the *t*-test ($p<0.001$, $T=-9.8$, $df=34$) which compared the absolute numbers of enterocytes per crypt irrespective of time. Figure 3.22b demonstrates that there was a slight increase in the number of enterocytes per crypt at day 14, but that at later time points the values were comparable with those of the control mice (GLM analysis indicated a significant change in both the intercept, $p<0.001$ and the slope, $p=0.006$). This difference can principally be attributed to the significant increase in the number of enterocytes per crypt at day 14 in inoculated mice ($p=0.001$, $T=4.74$, $df=9$) after which the values in inoculated animals return to normal. Notably there was a marked difference between the average number of enterocytes per crypt between infected WT and IFNγR$^{-/-}$ mice, with infected IFNγR$^{-/-}$ mice reaching a far higher cell average per crypt. *Post-hoc t*-test analysis demonstrated that the difference between these two populations at day 14 (the only point at which mice from both backgrounds were detectably infected) was significant ($p=0.004$, $T=-3.93$, $df=8$).

The average number of goblet cells per 100 enterocytes was also assessed and can be seen in Figure 3.23. GLM analysis illustrated that there was no significant difference in the intercept ($p=0.750$) between IFNγR$^{-/-}$ inoculated and control groups over time, but there was a significant difference in the slope ($p=0.038$).

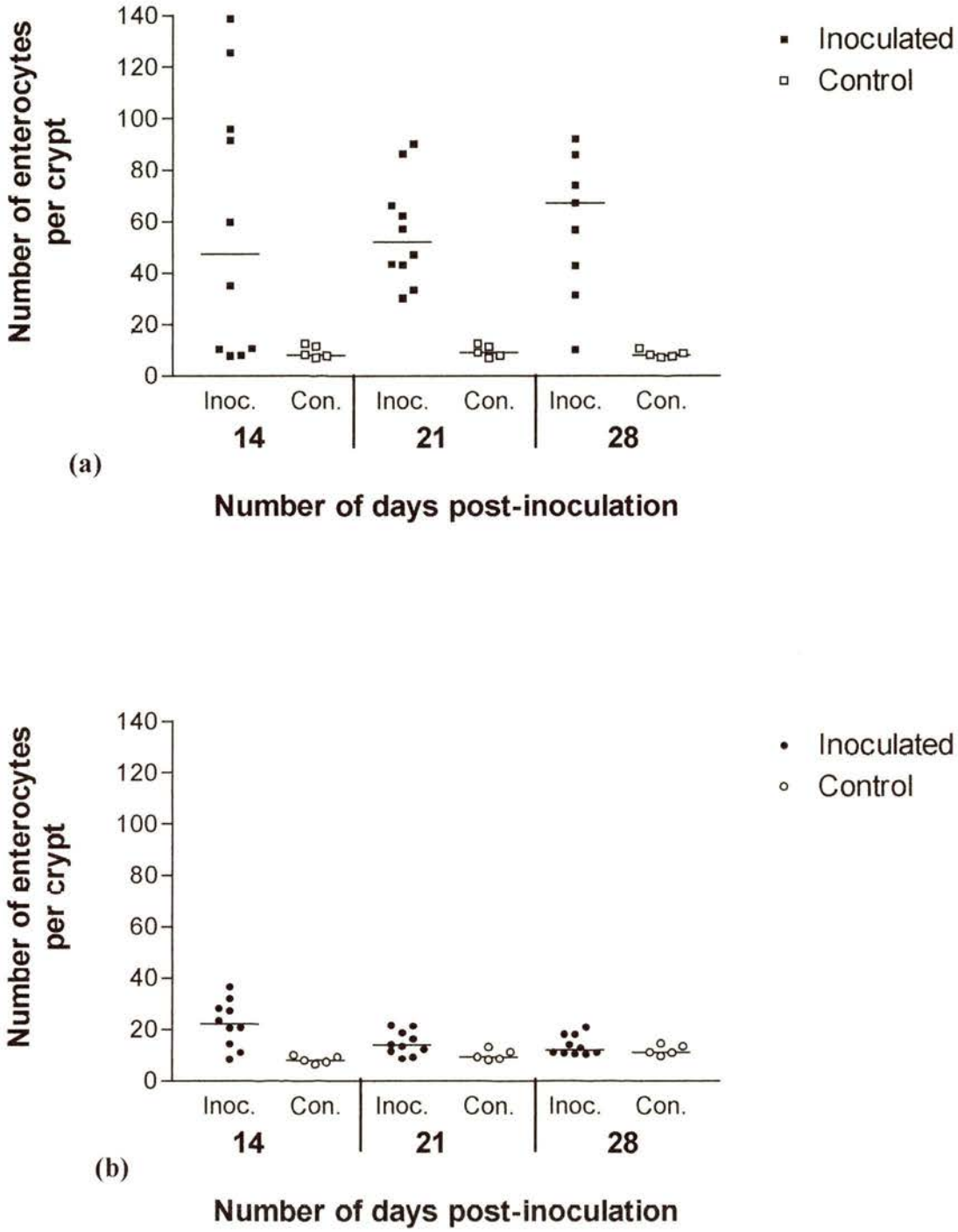


Figure 3.22. (a) IFNγR$^{-/-}$ and (b) WT mice.
Number of enterocytes per crypt in the colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and was determined by calculating the average number of cells over 10 random fields of view per section of colon).

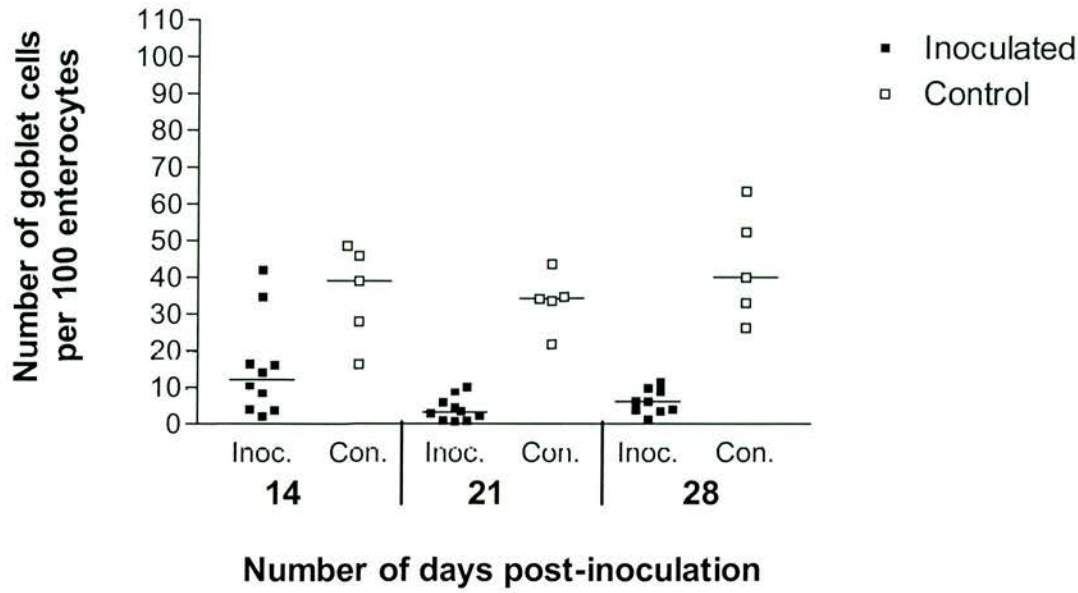


Figure 3.23a.

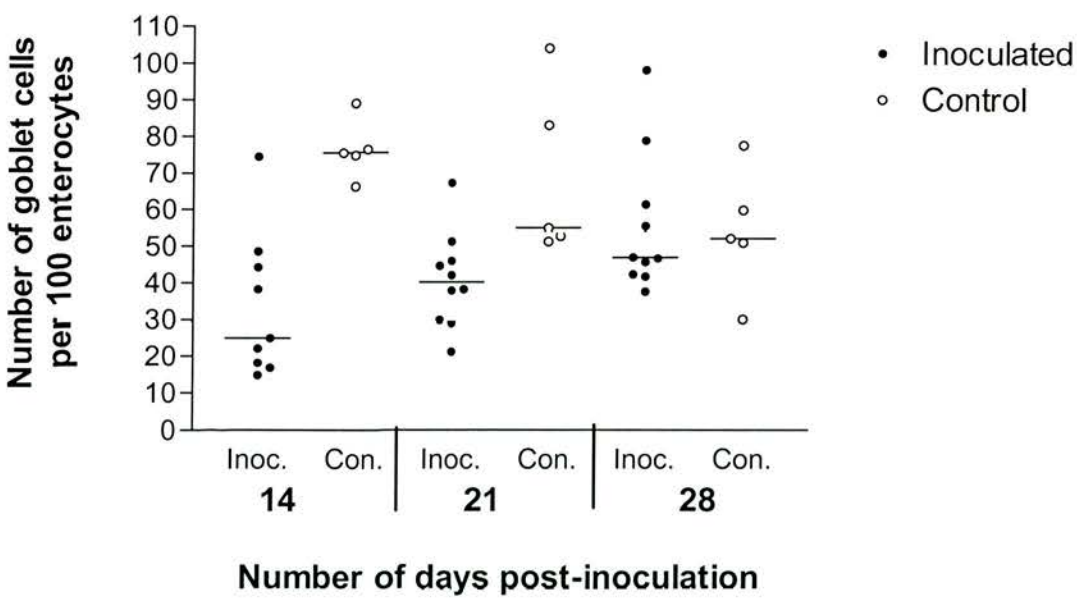


Figure 3.23b.

Figure 3.23. (a) IFNγR$^{-/-}$ and (b) WT mice.
Number of goblet cells per 100 enterocytes of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal and was determined by calculating the average cell count over 10 random fields of view per section.
Each control group contained 5 mice and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved and the first time point for the WT inoculated group).

The absence of significance for the intercept can probably be attributed to the two mice with an unusually high number of goblet cells at day 14 (one of which was not detectably infected, and the other was infected at a very low level). However, *post-hoc t*-test analysis demonstrated that there was a significant reduction in the actual number of goblet cells per 100 crypt enterocytes in inoculated IFNγR$^{-/-}$ mice in comparison to the control group ($p<0.001$; $T=-8.01$; $df=22$). The graph indicates a clear difference between the number of goblet cells in the two groups at all three time points. In contrast Figure 3.23b illustrates a changing pattern in the number of goblet cells per 100 enterocytes in the WT group inoculated with *L. intracellularis*. GLM analysis indicated that both the intercept ($p<0.001$) and slope ($p=0.002$) were significantly different between the inoculated and control groups. More specifically *post-hoc t*-test analysis demonstrated that at day 14 there were significantly less goblet cells per 100 enterocytes in the inoculated group ($p<0.001$; $T=-5.68$), but that this difference was lost by day 28 ($p=0.880$; $T=0.15$; $df=9$; mean value for inoculated mice=55.5 and the mean value for the control mice=54.0).

3.3.3 Presence of *L. intracellularis* detected by immunohistochemical staining.

Colon and ileum samples were examined microscopically for hyperplastic crypts in association with positive staining for *L. intracellularis*. The control mice for both wild type and IFNγR$^{-/-}$ mice were free of infection with *L. intracellularis* throughout the study. As shown in the previous section infection with *L. intracellularis* was associated with a reduction in the number of goblet cells (Figure 3.23) as well as an increase in the number of epithelial cells in infected crypts (Figure 3.22). Relative infection was assessed as the percentage of infected crypts (Figure 3.24).

Lesions of proliferative enteropathy were only evident at 14 days post-inoculation in WT animals. Of the 10 inoculated with *L. intracellularis* in this group, 9 presented with immunohistochemically detectable organisms within enterocytes of the colon and ileum.

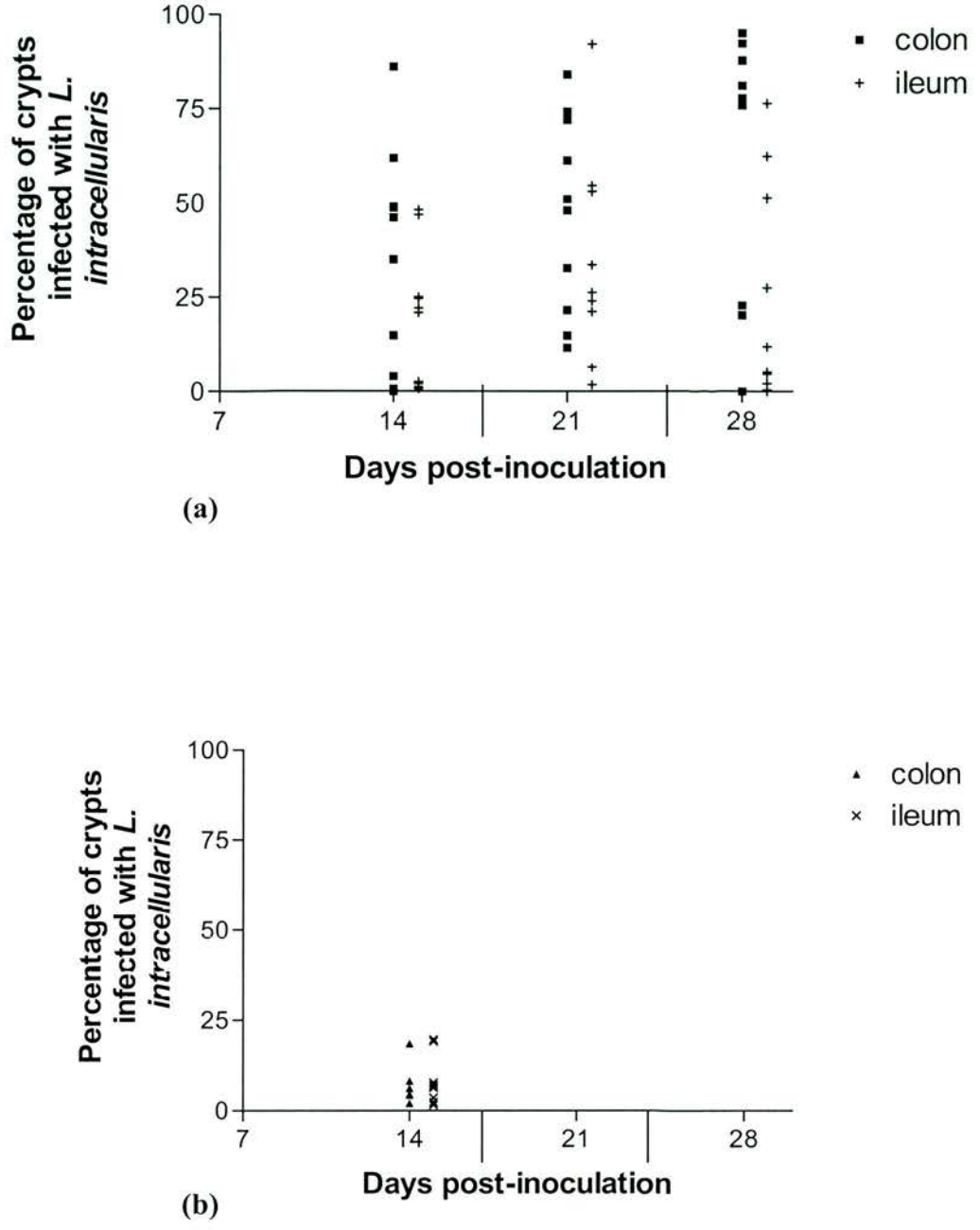


Figure 3.24. Percentage of crypts infected with *L. intracellularis* in all animals with lesions of proliferative enteropathy following inoculation with *L. intracellularis.* Control mice were given SPG only. (a), IFNγR$^{-/-}$ and (b) WT mice.
Each value represents the count for an individual animal (and is determined by calculating the average proportion of infected crypts in 10 random fields of view per section of intestine).

L. intracellularis could be detected in both the ileum and colon of 6 animals, and exclusively in the ileum of the remaining 3 animals. There was no evidence of *L. intracellularis* at either of the later time points. In contrast, 100% of IFNγR$^{-/-}$ mice inoculated with *L. intracellularis* developed lesions of proliferative enteropathy. At day 14 post-inoculation 8 animals were infected in both the colon and ileum, and the remaining 2 were infected in the ileum only. At day 21, 9 animals were infected in both the ileum and colon (the other animal was infected in the colon, but ileal samples were not retrieved). At day 28, 7 animals were positive immunohistochemically for *L. intracellularis* in both the colon and ileum, 2 in the ileum only, and 1 exclusively in the colon. However, logistic regression analyses performed demonstrated no significant relationship between the proportion of crypts infected with *L. intracellularis* and time (colon, X^2=2.093, df=1, p=0.148; ileum, X^2=0.142, df=1, p=0.706).

Figure 3.25 shows representative H+E and immunohistochemical results following inoculation with *L. intracellularis.*

Table 3.3 is a summary of all the challenge experiments and indicates the consistently higher proportion of IFNγR$^{-/-}$ animals infected in comparison to WT.

Experiment	7	14	21	28	35	40
1 129Sv/Ev	0/4	2/4	0/4	0/4	NT	0/2
IFNγR$^{-/-}$	0/4	1/4	3/4	2/3	NT	2/2
2 129Sv/Ev	NT	3/4	4/4	0/4	0/4	NT
IFNγR$^{-/-}$	NT	3/4	4/4	4/4	1/1	NT
3 129Sv/Ev	NT	3/4	0/4	0/4	0/3	NT
IFNγR$^{-/-}$	NT	4/4	4/4	6/6	2/2	NT
4 129Sv/Ev	NT	9/10	0/10	0/10	NT	NT
IFNγR$^{-/-}$	NT	10/10	10/10	10/10	NT	NT

NT = not tested.

Table 3.3. Number of mice positive for immunohistochemically detected *L. intracellularis* in either the ileum or colon.

Figure 3.25a.

Figure 3.25b.

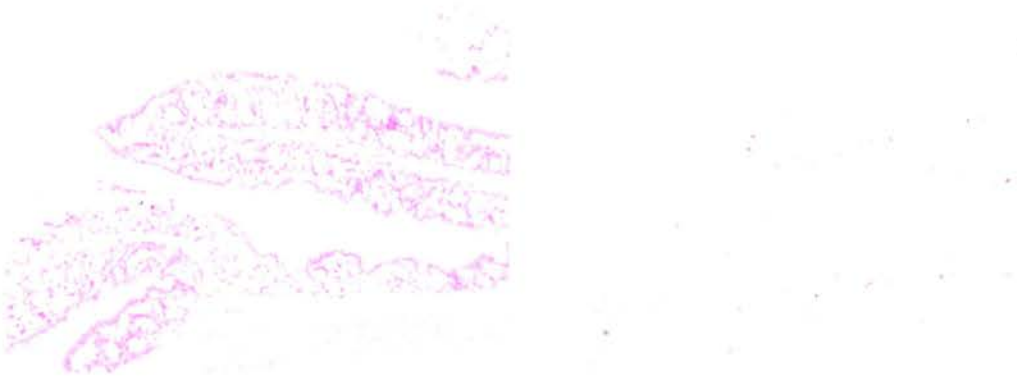


Figure 3.25c.

Figure 3.25. Representative H+E and anti-*L. intracellularis* staining on (a) IFNγR$^{-/-}$ and (b) WT mice inoculated with *L. intracellularis* and (c) WT control mice, all at day 14 post-inoculation. All pictures x100 magnification, inset for the WT mouse is x400 and illustrates an infected crypt with brown stained *L. intracellularis* evident in the apical cytoplasm.
Infected IFNγR$^{-/-}$ mice demonstrate epithelial hyperplasia in association with infection, and an associated loss of goblet cells.
Infected WT mice demonstrate mildly hyperplastic infected crypts, with goblet cells still evident.
Control mice demonstrate normal epithelial structure with scattered goblet cells.

3.3.4 Phenotyping studies.

Cell counts were assessed with ten areas of tissue selected and specific areas of interest delineated as described in section 2.3.2.10. Analysis of specific cellular infiltration was compartmentalised and measured either as the number of cells per mm^2 lamina propria (average area counted per section was $0.4mm^2$), or intraepithelial leukocytes were determined by counting positive cells in crypt epithelial regions, and results expressed as positive cells per 100 enterocytes (average number of enterocytes counted was 900 per section). Each population of infiltrating cells are outlined in turn and the final subsection, 3.3.4.9, provides a summary of the analyses performed.

3.3.4.1 CD11b.

$CD11b^+$ cells were measured as the number of infiltrating cells per mm^2 lamina propria. Figure 3.26 demonstrated that inoculated $IFN\gamma R^{-/-}$ mice had a slight increase in the infiltration of $CD11b^+$ cells at all time points (in comparison to the controls). Furthermore statistical analysis showed that the intercepts were significantly different ($p=0.049$), which indicated that there were a higher number of cells present in inoculated mice. In contrast no statistical significance could be ascribed to the slope ($p=0.433$), i.e. there was no significance in the variance of cell infiltration over time between the two groups. Analyses for the infiltration of $CD11b^+$ cells in inoculated WT mice demonstrated statistically significant changes in the both the intercept ($p=0.004$) and the slope ($p=0.021$). Figure 3.26b demonstrates that there was a slight increase in cell numbers at day 14, and then the values declined towards those of the controls.

Figure 3.27 illustrates representative pictures following CD11b immunohistochemical staining.

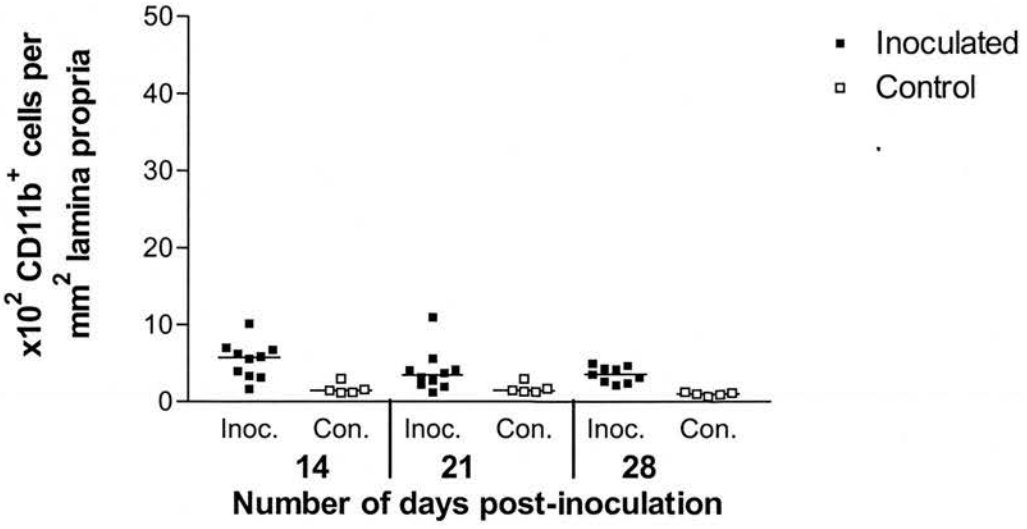


Figure 3.26a.

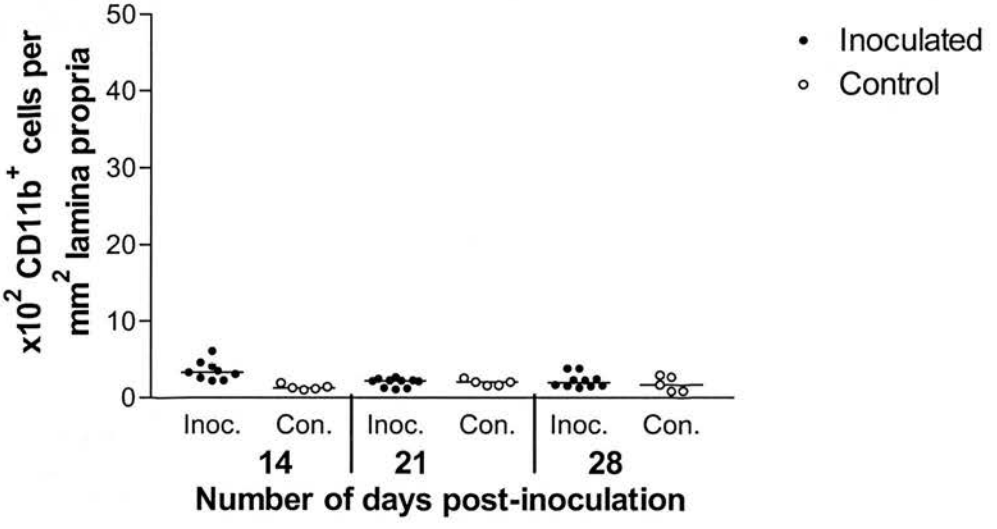


Figure 3.26b.

Figure 3.26. (a) IFNγR$^{-/-}$ and (b) WT mice.
CD11b^{+} cell infiltration per mm^{2} lamina propria of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and is determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contains 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

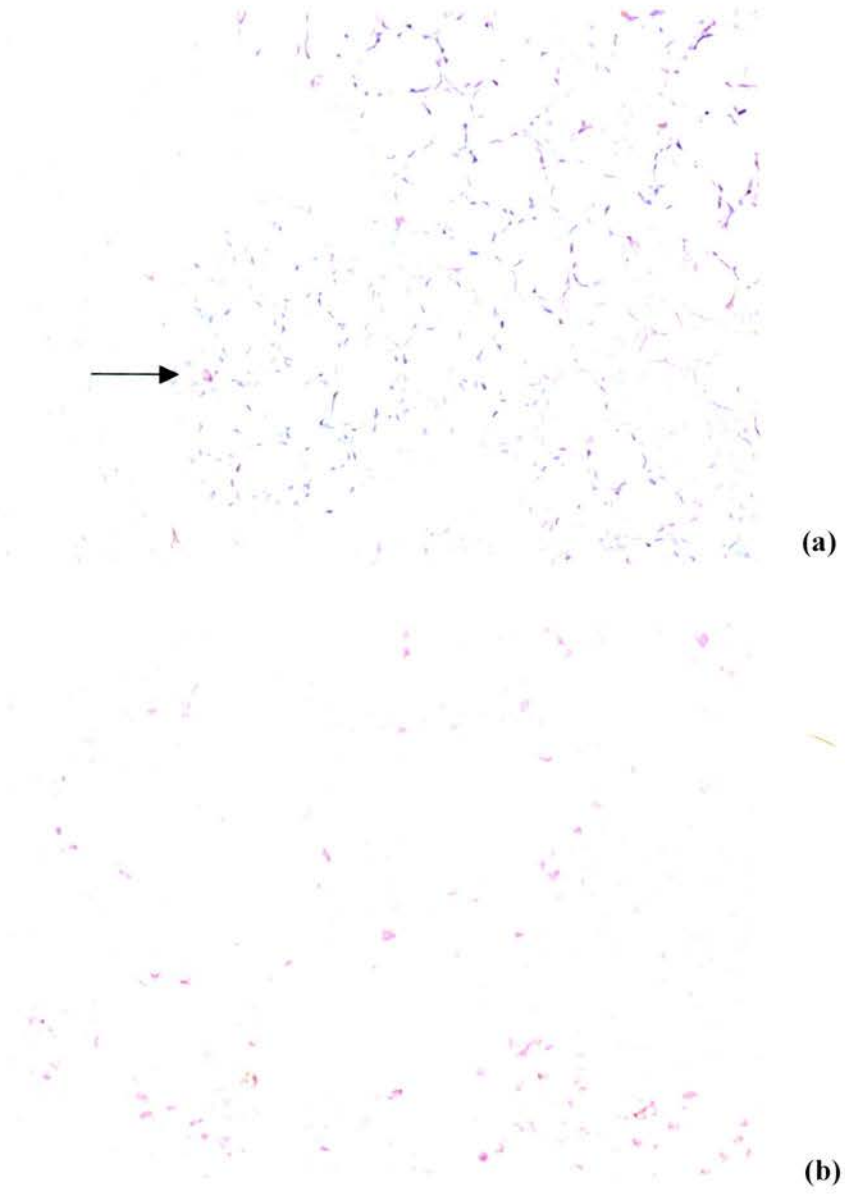


Figure 3.27. Representative sections of (a) control and (b) WT inoculated mice 14 days post-inoculation, x200.
Scattered $CD11b^+$ cells are evident in the lamina propria (marked by arrow).

3.3.4.2 CD3.

$CD3^+$ cells were detected immunohistochemically and counted as the number of infiltrating cells per mm^2 lamina propria, and the number of positive cells per 100 enterocytes (Figures 3.28 and 3.29).

In inoculated $IFN\gamma R^{-/-}$ mice there was an elevated $CD3^+$ cell count relative to control values at all three time points studied, but no statistical significance could be assigned to either the intercept or slope parameters ($p=0.068$ and $p=0.94$ respectively, section 3.3.4.9). If, however, the two animals with no detectable *L. intracellularis* at day 14 were removed from the analysis, then there was a significant difference in the intercept value ($p<0.05$), but not the slope. This indicates that there was a significantly higher number of infiltrating $CD3^+$ cells in the lamina propria of mice infected with *L. intracellularis* relative to the controls. The $CD3^+$ cell infiltration remains at a high level for all three time points, as does *L. intracellularis* infection.

In WT mice there was an increased infiltrating $CD3^+$ cell population in the lamina propria of mice inoculated with *L. intracellularis* (in contrast to the control values). This was apparent at all three time points with the greatest number of $CD3^+$ cells seen at day 14. The numbers then gradually decreased, towards the control values, as time progressed. The decreasing $CD3^+$ cell infiltration coincided with the absence of *L. intracellularis* infection (which was only seen at day 14). There was a significant difference in the cell numbers, and change in infiltration pattern, between the control and inoculated group over time ($p<0.001$ and $p=0.001$ respectively, section 3.3.4.9).

The second subdivision of infiltrating cells assessed were those in the $CD3^+$ intraepithelial cell compartment and were measured as the number of $CD3^+$ cells per 100 enterocytes (see Figure 3.29). The pattern of $CD3^+$ cellular infiltration varied among the two compartments assessed for the inoculated $IFN\gamma R^{-/-}$ mice. The lamina propria infiltration demonstrated a marked increase in cell numbers at all three time points. In contrast the number of infiltrating $CD3^+$ intraepithelial lymphocytes were lower than the control animals at day 14, and did not show signs of increase until days 21 and 28.

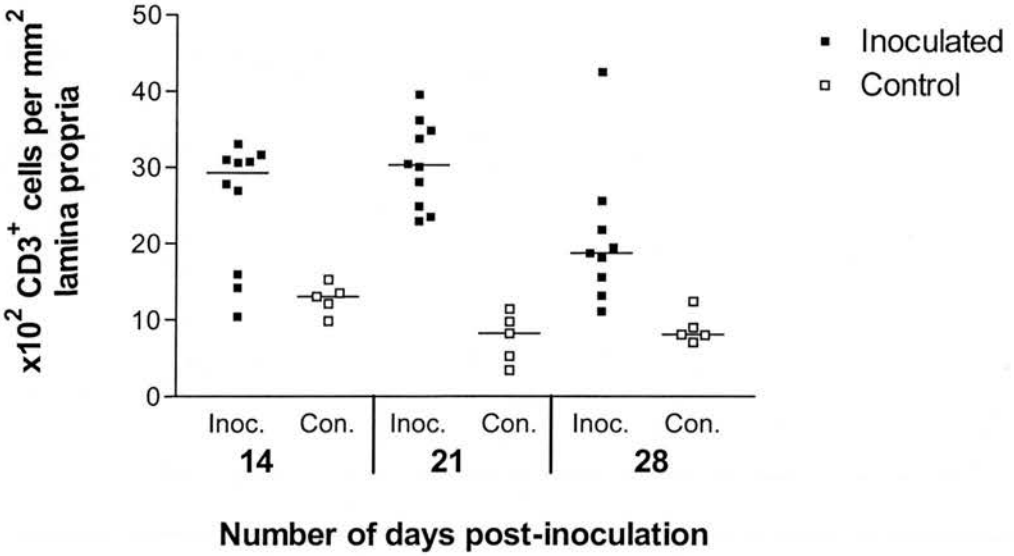


Figure 3.28a.

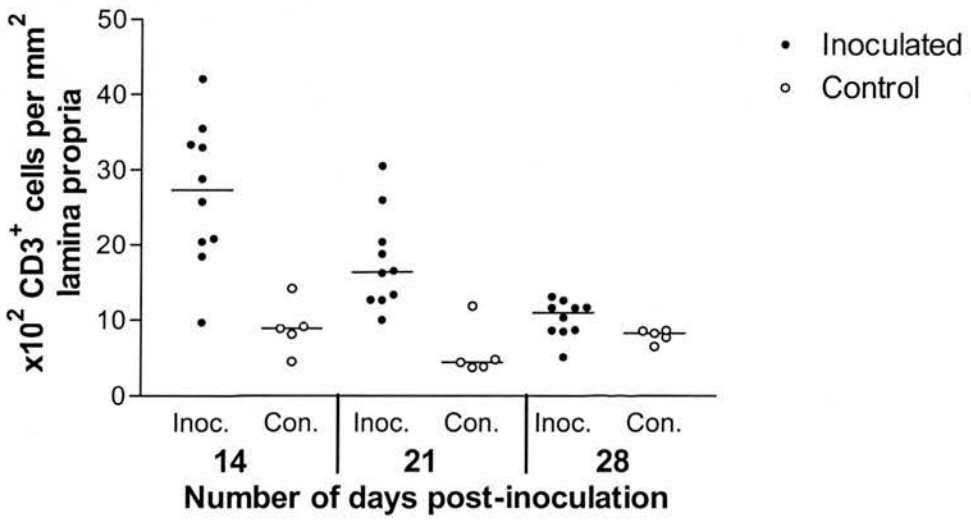


Figure 3.28b.

Figure 3.28. (a) IFNγR$^{-/-}$ and (b) WT mice.
CD3^{+} cell infiltration per mm^2 lamina propria of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and was determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contained 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

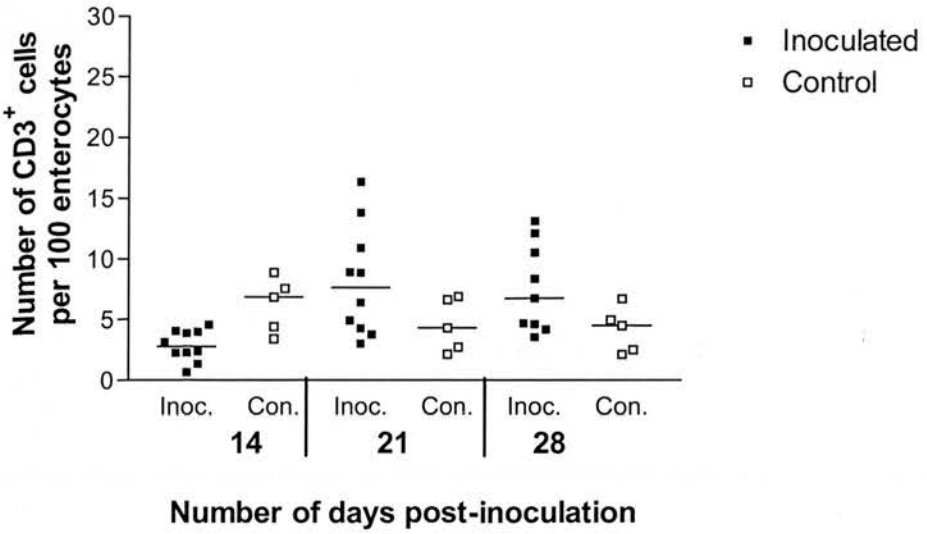


Figure 3.29a.

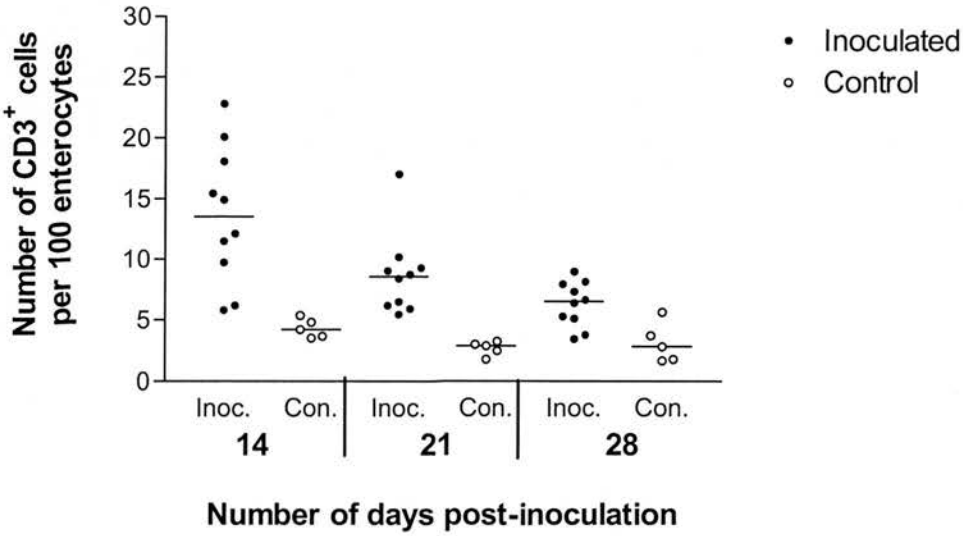


Figure 3.29b.

Figure 3.29. (a) IFNγR$^{-/-}$ and (b) WT mice.
CD3^{+} intraepithelial lymphocytes per 100 enterocytes of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and was determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contained 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

Section 3.3.4.9 shows that the values for the intercept were significantly different ($p=0.025$), but not that for the slope ($p=0.09$).

The pattern of infiltration seen for the WT mice was similar to that seen for the $CD3^+$ cell infiltration in the lamina propria. That is, at all time points there was an elevated cell count in comparison to the control values, and this was highest at day 14. Thereafter the levels declined towards the resting values for the control animals. These changes were statistically significant ($p<0.001$ and $p=0.02$ for the intercept and slope respectively, see section 3.3.4.9).

Due to the apparent differences between the infiltrating cell population of $CD3^+$ IELs at day 14 between the inoculated WT and $IFN\gamma R^{-/-}$ mice a *post-hoc* student *t*-test was performed on this set of data (incorporating only the animals with detectable *L. intracellularis*). This demonstrated that there was a significant difference ($p<0.05$) in the number of $CD3^+$ IELs at day 14 between the two backgrounds of mice.
The analysis was only performed at this single time point as it represented the only stage at which both WT and $IFN\gamma R^{-/-}$ mice displayed evidence of infection with *L. intracellularis.*

Figure 3.30 illustrates the association between infiltrating $CD3^+$ cells and proliferative crypts.

Figure 3.30a.

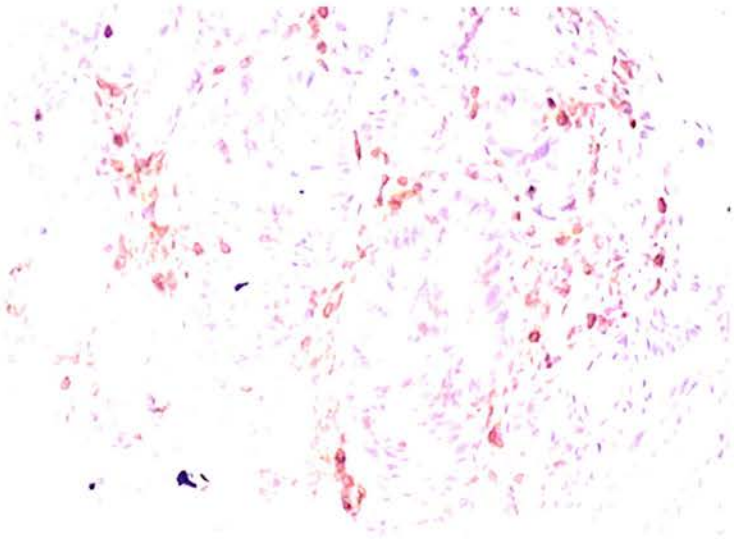


Figure 3.30b.

Figure 3.30. Immunohistochemical detection of $CD3^+$ cells in the colon of an $IFN\gamma R^{-/-}$ mouse inoculated with *L. intracellularis*, 14 days post-inoculation.
Figure 3.30a illustrates the association of $CD3^+$ cells with proliferative crypts, and demonstrates the distinction between adjacent areas of healthy and infected mucosa (x100).
Figure 3.30b is a higher magnification picture of the infected area (x200), showing $CD3^+$ cells in the lamina propria and the intraepithelial compartment.

3.3.4.3 CD4.

$CD4^+$ cells were measured as the number of infiltrating cells per mm^2 lamina propria (see Figures 3.31 and 3.32). $CD4^+$ cell infiltration was elevated in inoculated $IFN\gamma R^{-/-}$ mice at all three time points in comparison to the controls. There was a significant change in the rate of cell infiltration ($p=0.007$), see section 3.3.4.9), with the control values remaining relatively constant, but the $CD4^+$ population increasing as time progressed. No statistical significance could be attributed to the values for the intercept though ($p=0.771$), due to the low degree of infiltration for two animals with no detectable *L. intracellularis* at day 14. *Post-hoc* analysis removing these two aspects of the data, however, did assign significance to the overall number of infiltrating cells ($p<0.05$), and fits in with the trend displayed in Figure 3.31a.

WT mice demonstrated the highest $CD4^+$ cell count at 14 days post-inoculation, with the numbers then decreasing as time progressed (although always remaining higher than the control values), a similar pattern to that seen for $CD3^+$ lamina propria lymphocytes. This variation over time between the inoculated and control groups was statistically significant (for both the intercept, $p<0.001$ and the slope, $p<0.001$, see section 3.3.4.9).

Figures 3.28 and 3.31 confirm that the majority of $CD3^+$ cells infiltrating into the lamina propria following infection with *L. intracellularis* were also $CD4^+$, for both WT and $IFN\gamma R^{-/-}$ mice.

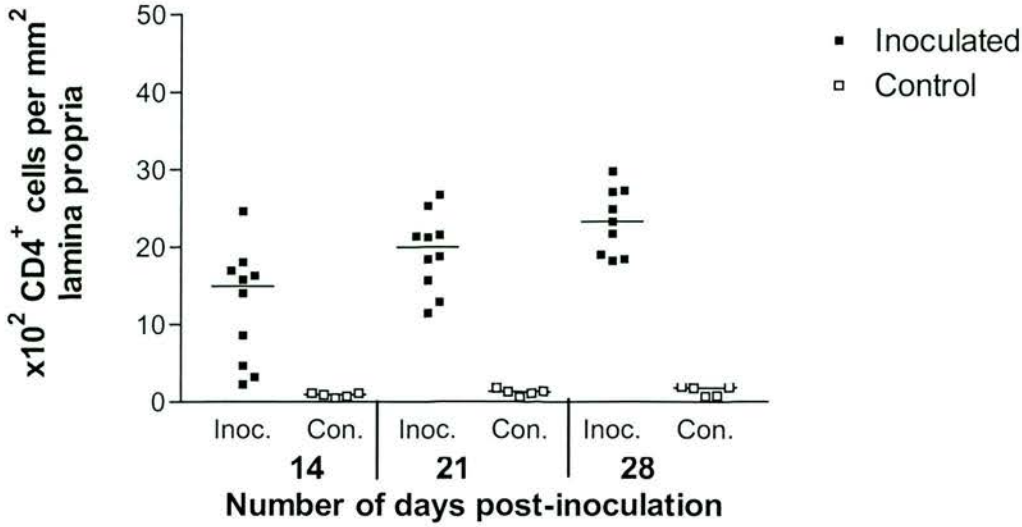


Figure 3.31a.

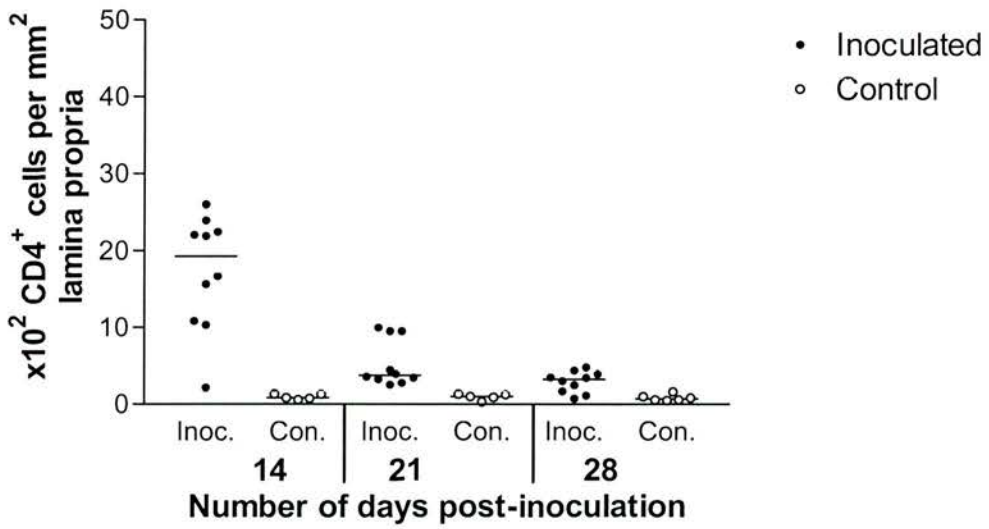


Figure 3.31b.

Figure 3.31. (a) IFNγR$^{-/-}$ and (b) WT mice.
CD4^{+} cell infiltration per mm^2 lamina propria of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and is determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contains 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

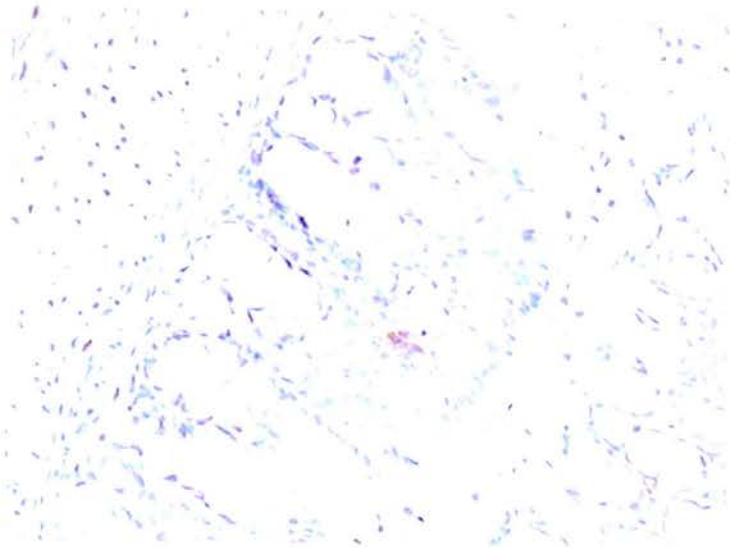


Figure 3.32a.

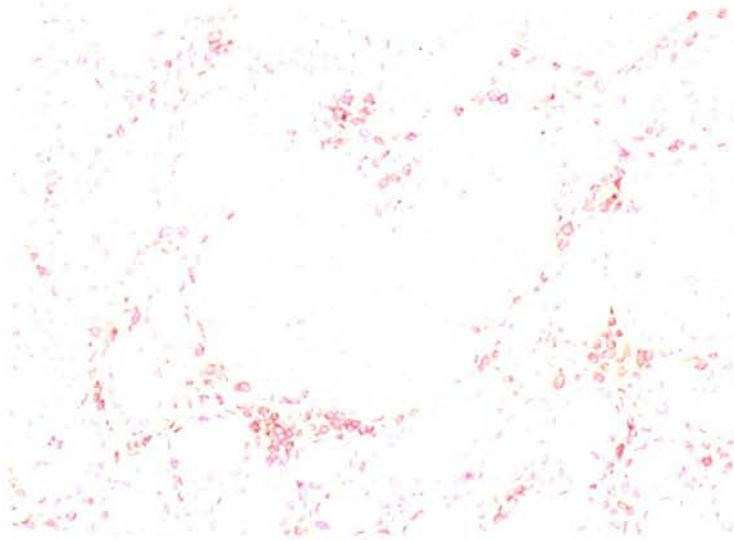


Figure 3.32b.

Figure 3.32. $CD4^+$ immunohistochemical staining illustrating a single positive cell in the lamina propria of a control mouse at day 14 (a), and a large infiltration of cells surrounding a proliferative crypt in the colon of an $IFN\gamma R^{-/-}$ mouse inoculated with *L. intracellularis* (b), also 14 days post-inoculation.

3.3.4.4 CD8.

$CD8^+$ cell infiltration was compartmentalised and assessed as the number of infiltrating cells per mm^2 lamina propria (Figures 3.33), and the number of $CD8^+$ intraepithelial cells per 100 enterocytes (Figure 3.34).

While $CD8^+$ cell infiltration was greater in inoculated rather than control $IFN\gamma R^{-/-}$ mice at all time points (Figure 3.33a), no statistical significance was assigned (intercept, $p=0.998$ and slope, $p=0.174$, section 3.3.4.9). The degree of $CD8^+$ cellular infiltration in the lamina propria did not show notable signs of increase until days 21 and 28 post-inoculation, and the cells appeared to take longer to migrate to areas of infection.

In comparison the $CD8^+$ cell population in the WT mice peaked at day 14 and then declined towards resting control values as time progressed. Comparison between Figures 3.28 and 3.33 again illustrates that the majority of cells infiltrating into the colonic lamina propria are $CD4^+$, rather than $CD8^+$. The cell infiltration did, however, follow a similar pattern to $CD4^+$ cells in WT mice with the highest level of cell infiltration seen at day 14 and coinciding with the presence of *L. intracellularis*. There was a statistically significant difference (section 3.3.4.9) for both the numbers of infiltrating cells involved (intercept, $p<0.001$), and the change in the pattern of infiltration (slope, $p=0.008$) over time.

The second compartment analysed was the infiltration of intraepithelial $CD8^+$ cells (Figure 3.34). The data following inoculation in $IFN\gamma R^{-/-}$ mice presents with a similar pattern to that seen for both $CD8^+$ lamina propria infiltration, and $CD3^+$ intraepithelial lymphocytes. That is at day 14 there are significantly lower numbers of infiltrating cells in $IFN\gamma R^{-/-}$ mice infected with *L. intracellularis,* in comparison to WT mice (determined by *a post-hoc* student *t*-test in all three cases, $p<0.05$). Also in $IFN\gamma R^{-/-}$ mice the infiltrating $CD8^+$ cells (both lamina propria and intraepithelial lymphocytes) do not demonstrate a notable increase in numbers until day 21 or 28 post-inoculation, unlike the $CD4^+$ lymphocytes, suggesting that a lag period in cell infiltration exists for these cells and they take longer to establish themselves at the site of infection.

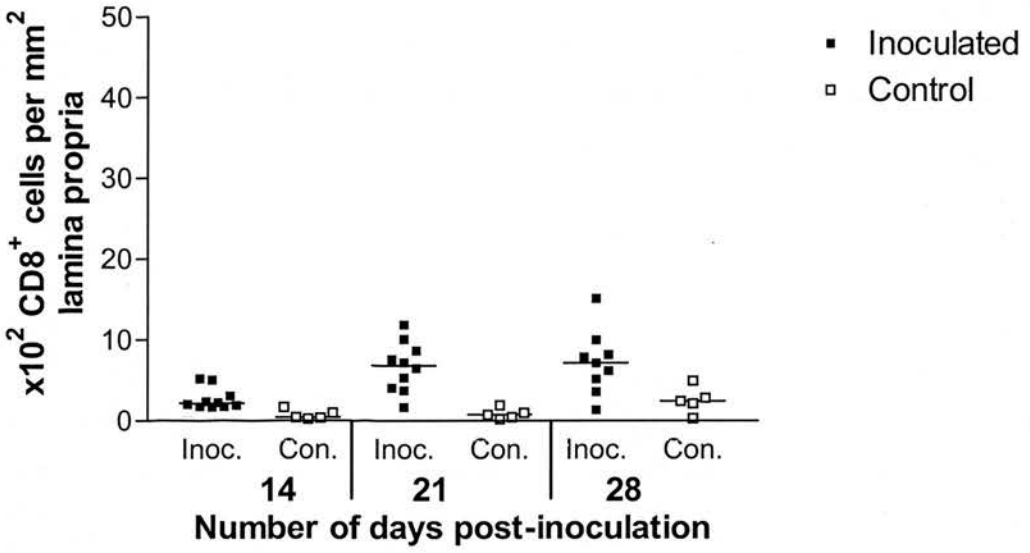


Figure 3. 33a.

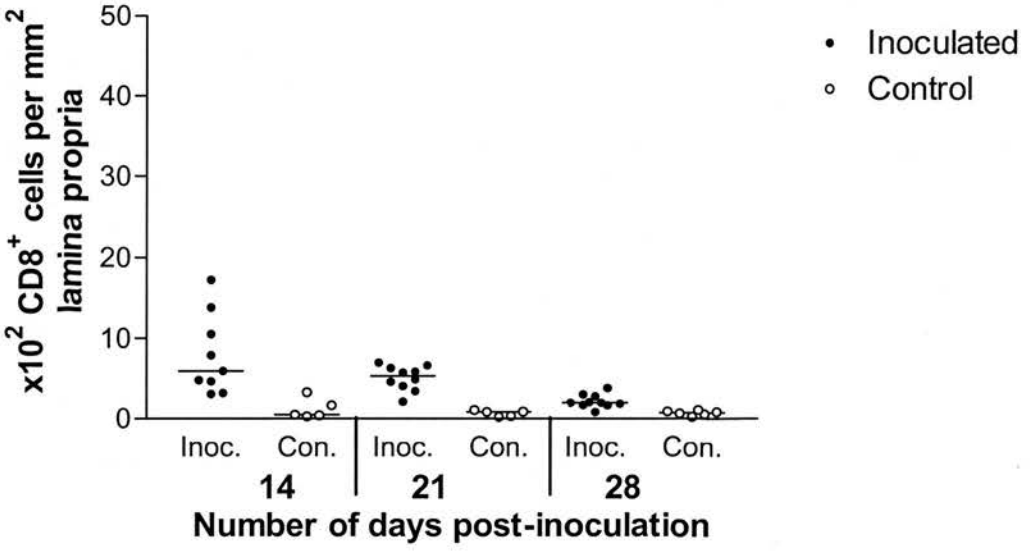


Figure 3.33b.

Figure 3.33. (a) IFNγR$^{-/-}$ and (b) WT mice.
CD8$^+$ cell infiltration per mm^2 lamina propria of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and is determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contains 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

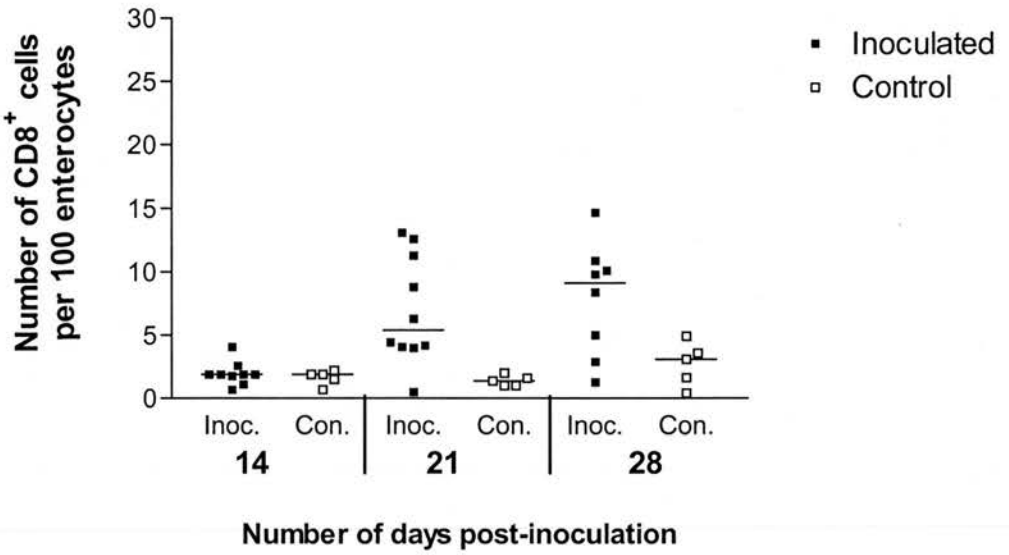


Figure 3.34a.

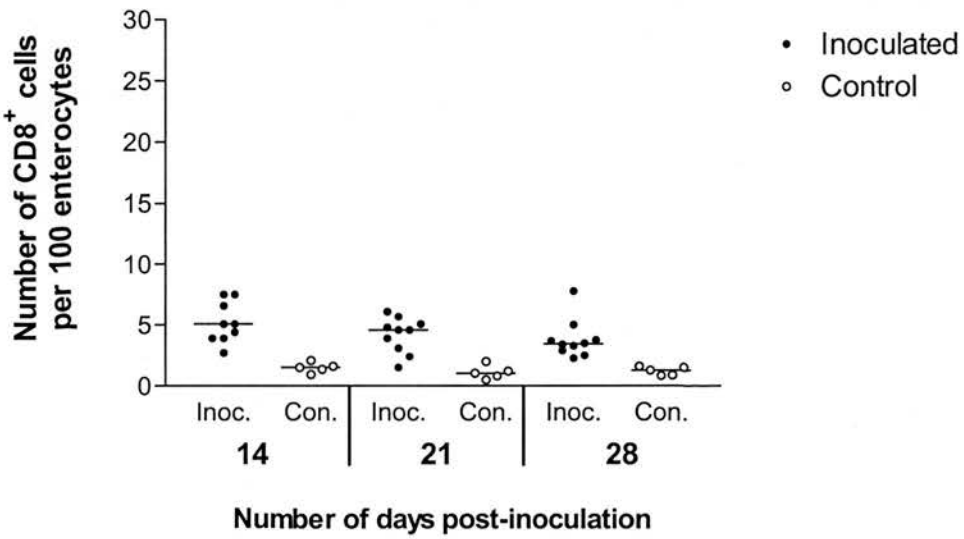


Figure 3.34b.

Figure 3.34. (a) IFNγR$^{-/-}$ and (b) WT mice.
$CD8^+$ intraepithelial lymphocytes per 100 enterocytes of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and was determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contained 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

In WT mice inoculated with *L. intracellularis* there was a significant difference in the number of infiltrating cells (intercept, p=0.005), but the slope was not significantly altered between the control and inoculated populations (p=0.297, Figure 3.34b). The cell numbers remained at a slightly elevated level in all animals inoculated with *L. intracellularis.*

Figure 3.35 is a representative photograph of infiltrating $CD8^+$ intraepithelial lymphocytes in both $IFN\gamma R^{-/-}$ and WT mice inoculated with *L. intracellularis.*

3.3.4.5 CD103.

$CD103^+$ cells were measured as the number of infiltrating cells per mm^2 lamina propria. Figure 3.36 demonstrates that following inoculation with *L. intracellularis* there was no significant change in the rate of infiltration of $CD103^+$ lymphocytes into the lamina propria (in either the WT or $IFN\gamma R^{-/-}$ mice) over time (p=0.971 and p=0.403 respectively). The data also illustrates that the cell numbers following inoculation (for both backgrounds of mice) were not significantly different from the control values obtained (WT, p=0.702, and $IFN\gamma R^{-/-}$, p=0.93, see section 3.3.4.9).
Figure 3.37 illustrates $CD103^+$ staining in sections of colonic mucosa.

3.3.4.6 E-cadherin.

Clear and unequivocally strong staining of normal epithelium was used as an internal positive control in all reactions against which the degree of expression was assessed (Sloncova et al., 2001). Strong granular staining was observed at cell-to-cell borders of epithelial tissues. The expression of E-cadherin varies among animals and even within areas in some cases, but there was evidence of E-cadherin staining in all WT and $IFN\gamma R^{-/-}$ mice whether inoculated or control. Figure3.38 illustrates strong E-cadherin staining in the proliferative crypts of an infected $IFN\gamma R^{-/-}$ mouse, as well as that seen in an inoculated (but non-infected) WT, both at day 21 post-inoculation.

Figure 3.35a.

Figure 3.35b.

Figure 3.35. $CD8^+$ cells detected immunohistochemically.
Figure 3.35a illustrates an $IFN\gamma R^{-/-}$ mouse 28 days post-inoculation with *L. intracellularis*, the time point at which $CD8^+$ IELs reached their maximum in $IFN\gamma R^{-/-}$ mice (x200).
Figure 3.35b illustrates a WT mouse 14 days post-inoculation with *L. intracellularis*, the time point at which $CD8^+$ cells reached their maximum in WT mice (x400).
Both figures illustrate that a large proportion of $CD8^+$ cells are found in the intraepithelial compartment in comparison to the lamina propria.

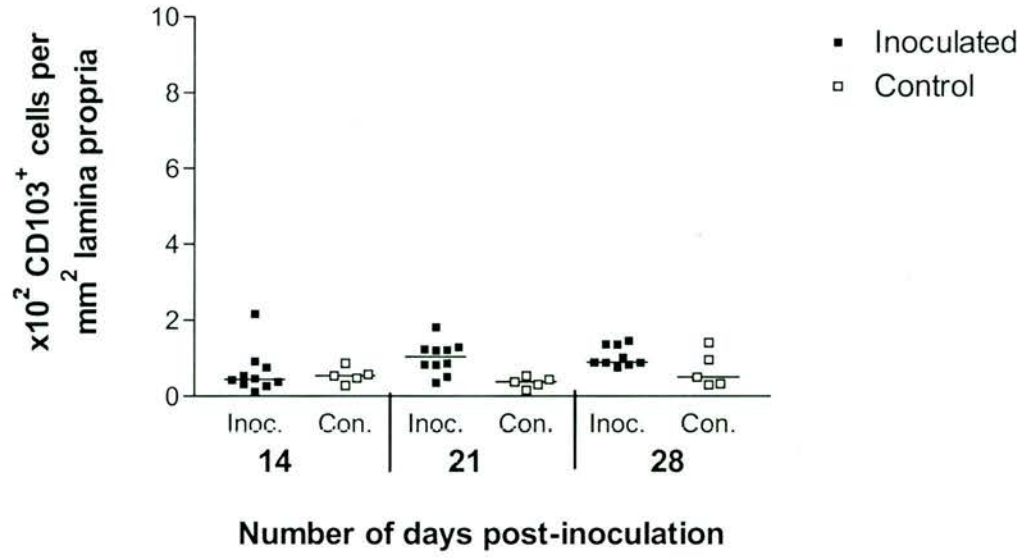


Figure 3.36a.

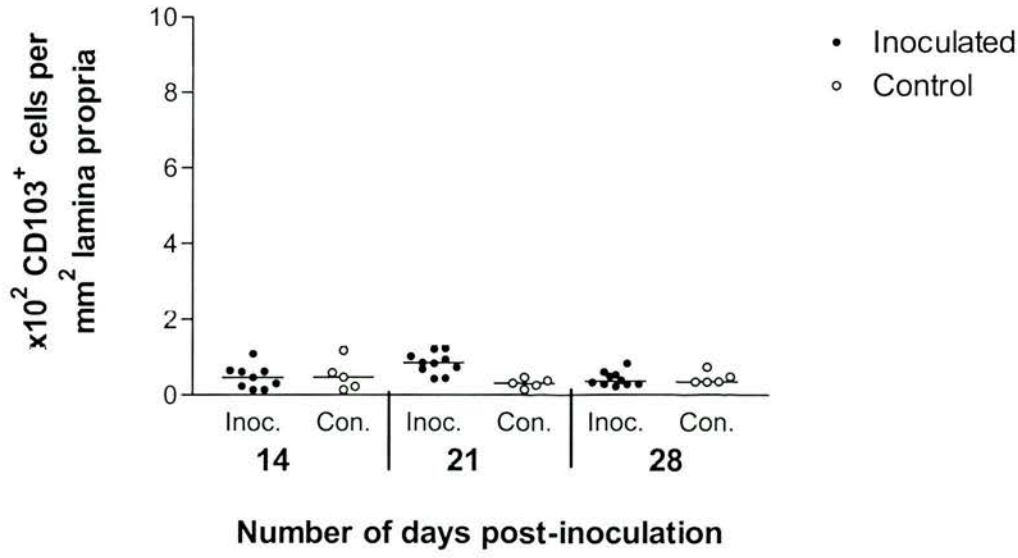


Figure 3.36b.

Figure 3.36. (a) IFNγR$^{-/-}$ and (b) WT mice.
CD103^{+} cell infiltration per mm^2 lamina propria of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and is determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contains 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).
Note the change in scale of the y axis due to the smaller numbers of cells detected.

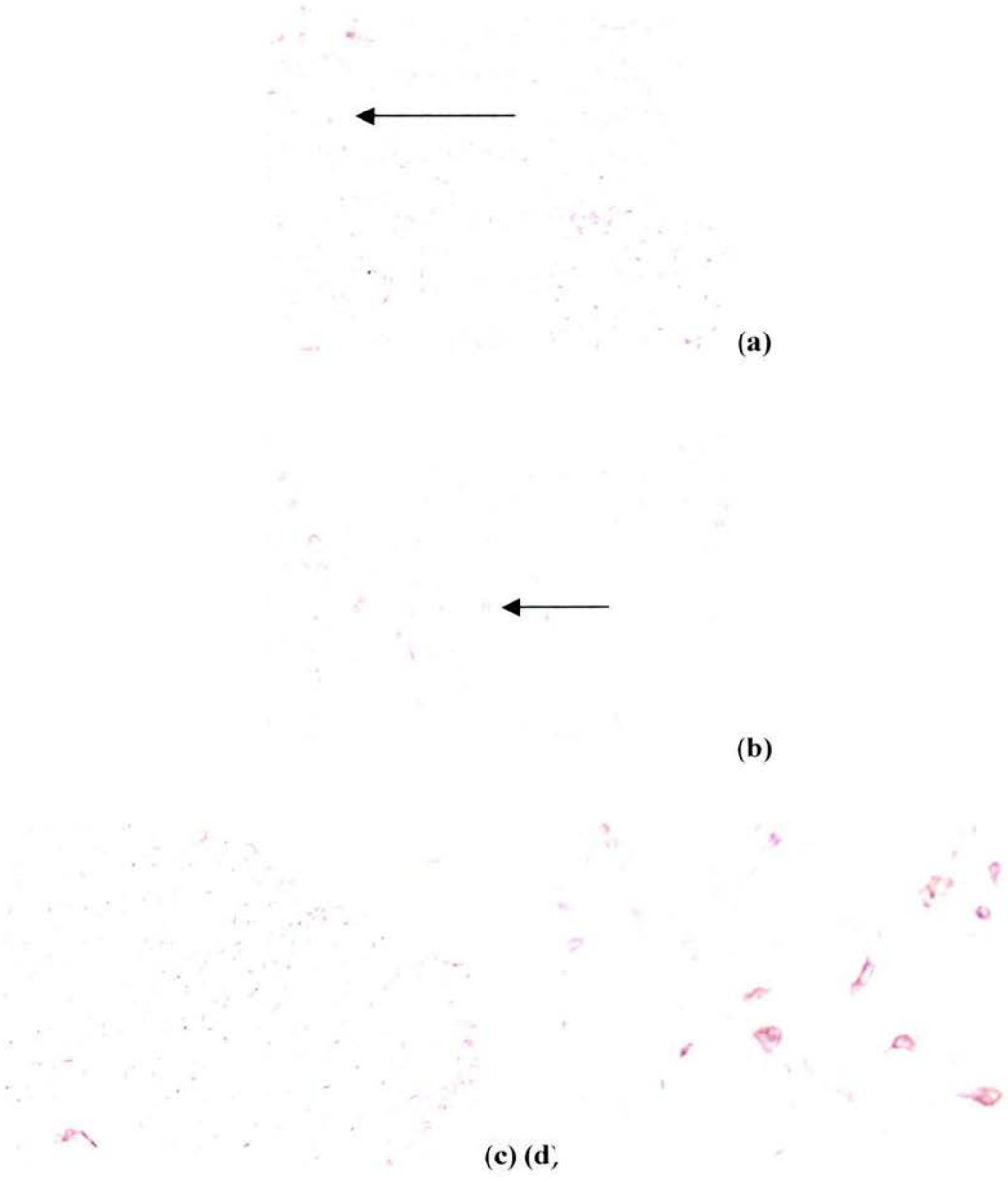
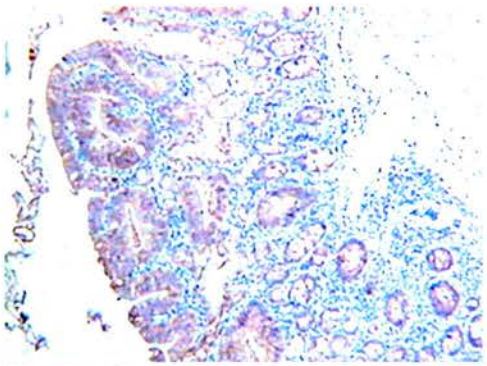
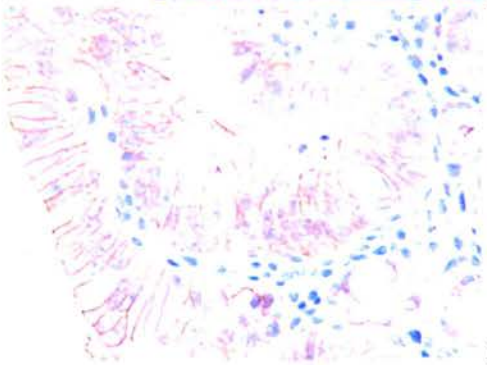


Figure 3.37. CD103^{+} cells, day 21 post-inoculation.
(a) Control (IFNγR$^{-/-}$), x200
(b) IFNγR$^{-/-}$, x200
(c) WT, x100 and (d), higher magnification, x400 demonstrating IELs.

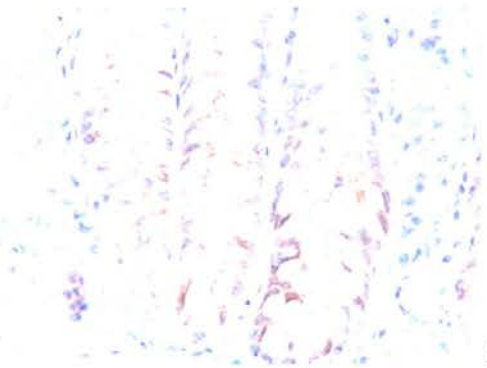


(a)



(b)

(c)



(d)

Figure 3.38. E-cadherin detected immunohistochemically on both IFNγR$^{-/-}$ (a, b) and WT mice (c, d) inoculated with *L. intracellularis*.
Figures a and c are x100, and figures b and d x400 magnification to illustrate the presence of E-cadherin surrounding the epithelial cells.

3.3.4.7 γδ TCR.

γδ TCR^+ cells were measured as the number of infiltrating cells per mm^2 lamina propria (Figure 3.39). Following inoculation with *L. intracellularis* there were no apparent changes in the infiltration of γδ TCR^+ lymphocytes into the lamina propria (in the WT or $IFN\gamma R^{-/-}$ mice, p=0.225 and p=0.6 respectively) as time progressed. The data also illustrates that the cell numbers following inoculation (for both backgrounds of mice) were not significantly different from the control values obtained (WT, p=0.302 and $IFN\gamma R^{-/-}$, p=0.883, see section 3.3.4.9).

Figure 3.40 illustrates representative photographs following immunohistochemical detection of γδ TCR^+ cells.

3.3.4.8 Plasma cells.

Figure 3.41 demonstrates positive B220 staining in the colonic lymphoid follicle of an $IFN\gamma R^{-/-}$ mouse inoculated with *L. intracellularis*. It demonstrates that although there was clear staining in this region of the intestinal tract, there were very few positive cells within the lamina propria. Consequently an histochemical technique was employed to stain plasma cells so that an aspect of B cell involvement in the immune response could be enumerated in this way.

Plasma cells were measured as the number of infiltrating cells per mm^2 lamina propria (Figures 3.42 and 3.43). Following inoculation with *L. intracellularis* there was a gradual increase in the number of plasma cells in the lamina propria of $IFN\gamma R^{-/-}$ mice over the course of the study, and this was statistically significant (p=0.008). The value for the intercept was not significant however (p=0.306), indicating that the accumulation of B cells to the site of infection occurred several weeks after inoculation. In contrast to this there were no significant changes in the degree of plasma cell infiltration in WT mice for either the intercept (p=0.272) or the slope (p=0.942).

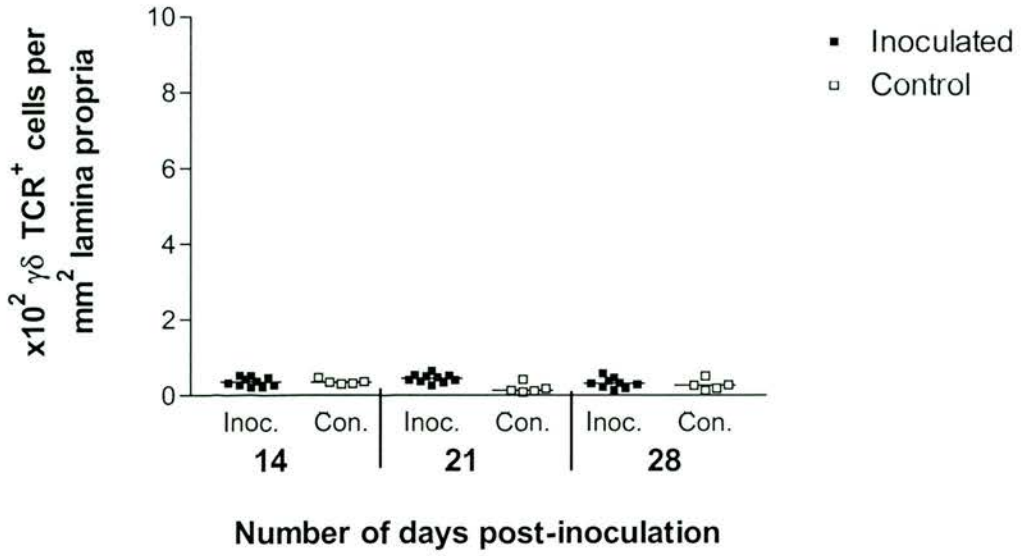


Figure 3.39a.

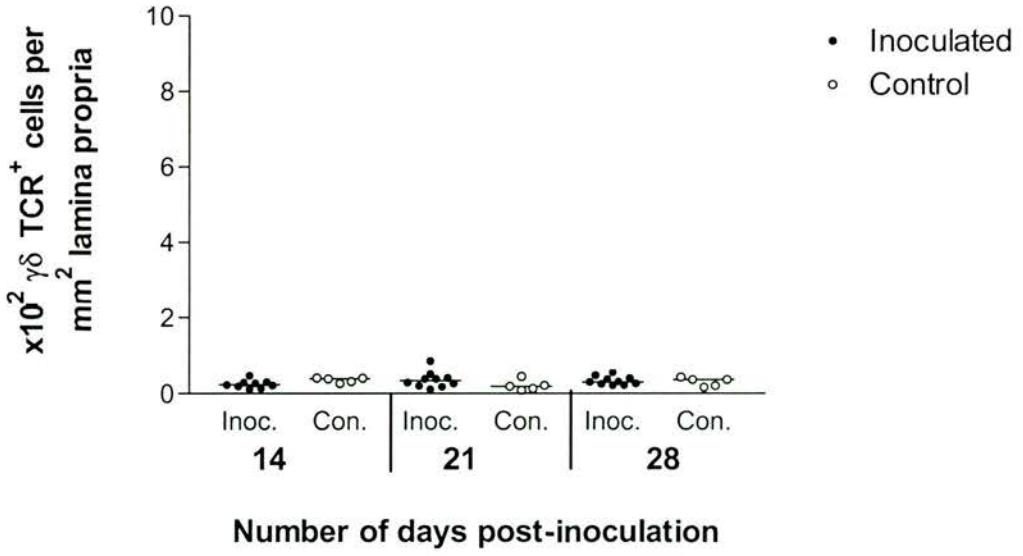


Figure 3.39b.

Figure 3.39. (a) IFNγR$^{-/-}$ and (b) WT mice.
γδ TCR^{+} cell infiltration per mm^2 lamina propria of colonic mucosa, following inoculation with either *L. intracellularis.* Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and is determined by calculating the average cell infiltration of 10 random fields of view per section of colon).
Each control group contains 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).
Note the change in scale of the y axis due to the smaller numbers of cells detected.

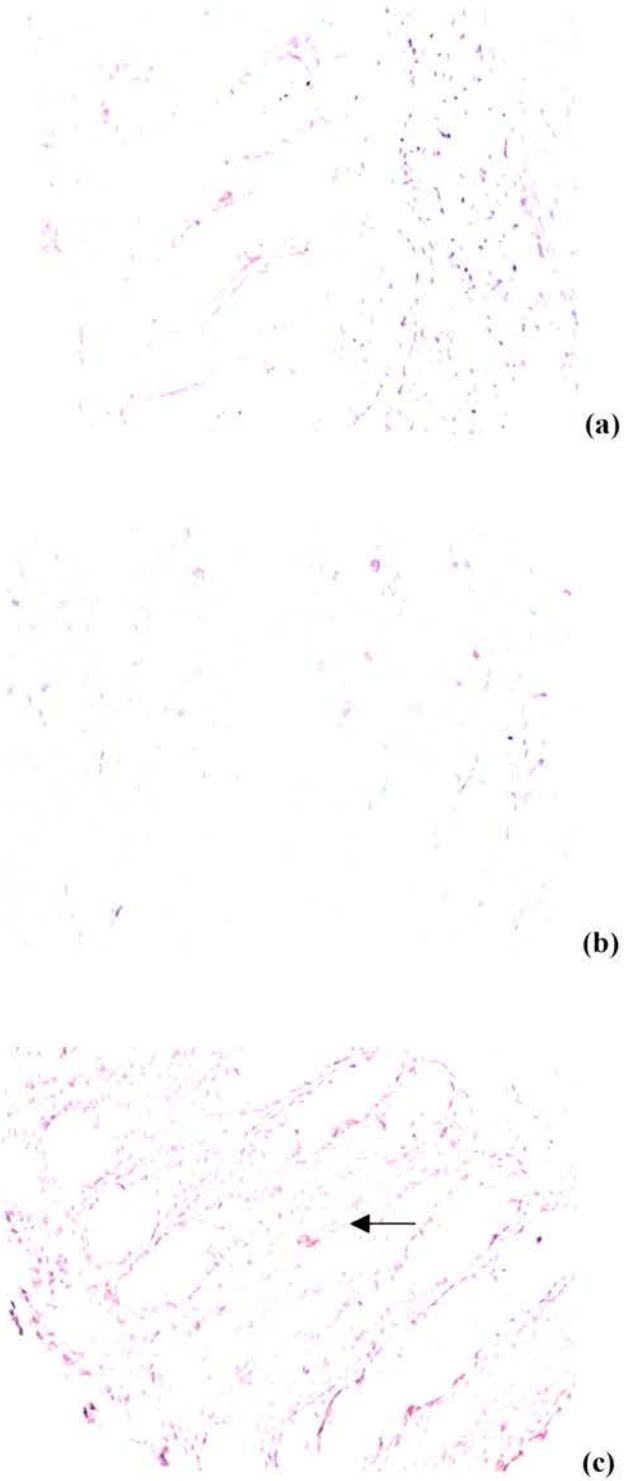


Figure 3.40. γδ TCR^{+} cells, typical representative illustrations at various time points post-inoculation illustrating intraepithelial lymphocytes as well as lamina propria lymphocytes.
(a) control (WT),
(b) $IFN\gamma R^{-/-}$,
(c) WT.

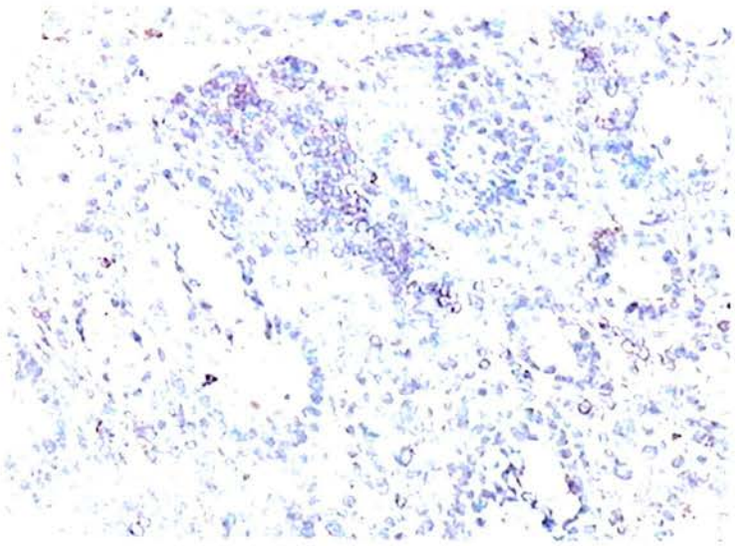


Figure 3.41a.

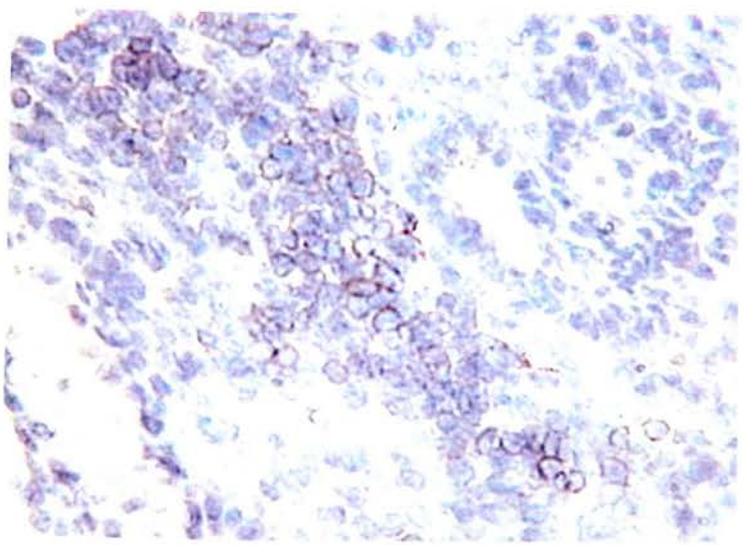


Figure 3.41b.

Figure 3.41. $B220^{+}$ staining clearly seen in the lymphoid follicle of a representative $IFN\gamma R^{-/-}$ mouse, but no cells apparent within the colonic lamina propria.
Figure (a) is at x250 magnification, and (b) is at x400.

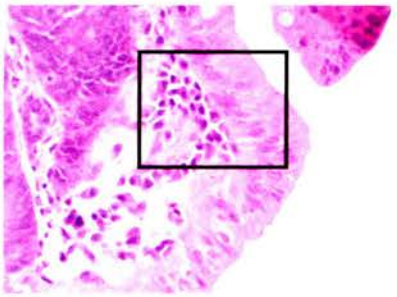


Figure 3.42a.

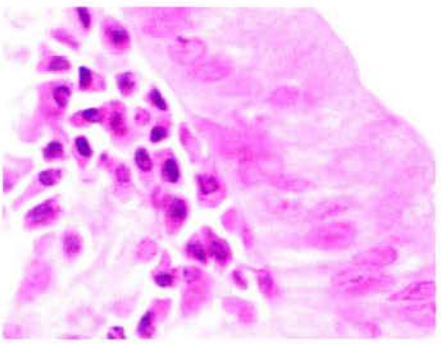


Figure 3.42b.

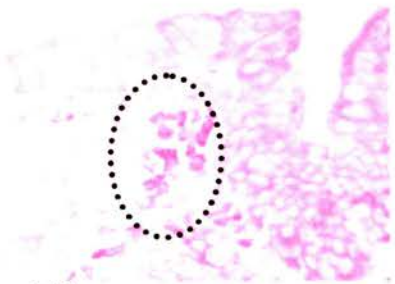


Figure 3.42c.

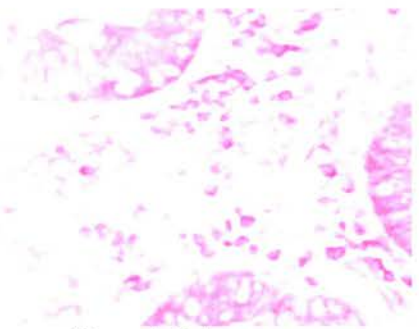


Figure 3.42d.

Figure 3.42.
Sections of colon from infected IFNγR$^{-/-}$ mouse 28days post inoculation.
Figure 3.42a shows large cluster of plasma cells beneath the surface epithelium. H&E x250.
Figure 3.42b enlarges the area highlighted in Figure 3.42a to demonstrate the characteristic plasma cell morphology.
Figure 3.42c shows a similar aggregate of plasma cells (within oval area) beneath the surface epithelium highlighted with the stain methyl green pyronin (MGP) which stains plasma cell cytoplasm red.
Figure 3.42d shows large numbers of plasma cells in the lamina propria between hyperplastic crypts (MGP).

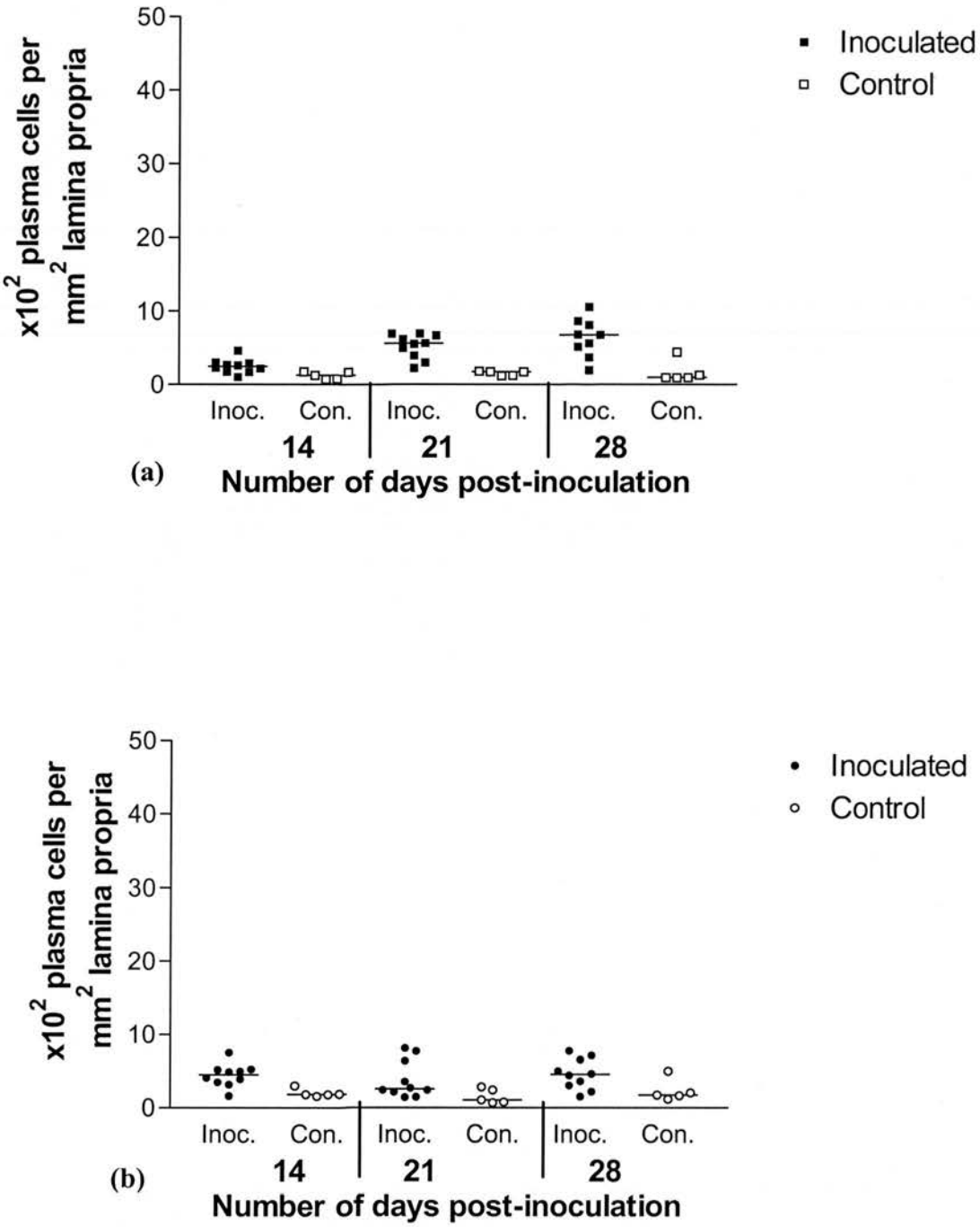


Figure 3.43. (a) IFNγR$^{-/-}$ and (b) WT mice.
Plasma cell infiltration per mm^2 lamina propria of colonic mucosa, following inoculation with *L. intracellularis*. Control groups were inoculated with SPG buffer only.
Each point represents the count for an individual animal (and was determined by calculating the average cell infiltration of 10 random fields of view per section).
Each control group contains 5 mice, and each inoculated group 10 mice (with the exception of the final time point for the IFNγR$^{-/-}$ group where one mouse died and suitable tissue for analysis was not retrieved).

3.3.4.9 Statistical analysis.

This section summarises the analyses presented in the previous sections (see Materials and Methods for descriptions of statistical tests used). The aim of this section is to bring together the statistical information and interpret the results in the general context of multiple cellular infiltration patterns.

Table 3.4 presents a summary of the cellular infiltration slope and intercept p values (see section 2.3.2.11) from the general linear models for all cell types considered.

Factor (for IFNγR$^{-/-}$)	p value	
	Intercept	Slope
CD3	0.068	0.94
CD4	0.771	**0.007**
CD8	0.998	0.174
CD103 (log)	0.93	0.403
γδ TCR	0.883	0.6
CD11b	**0.049**	0.433
Plasma cells	0.306	**0.008**
CD3 IEL	**0.025**	0.09
CD8 IEL	0.370	0.057

Factor (for WT)	p value	
	Intercept	Slope
CD3	**<0.001**	**0.001**
CD4	**<0.001**	**<0.001**
CD8 (log)	**<0.001**	**0.008**
CD103	0.702	0.971
γδ TCR	0.302	0.225
CD11b (log)	**0.004**	**0.021**
Plasma cells	0.272	0.942
CD3 IEL	**<0.001**	**0.02**
CD8 IEL	**0.005**	0.297

Table 3.4. P values associated with the cellular infiltration intercepts and slopes for all cell types assessed. Significant p values (<0.05) shown in bold. Log intercept and slope p values were used where indicated (see section 2.3.2.11).

The majority of factors for wild-type mice were associated with significantly different slopes and intercepts, with only one (for the infiltration of $CD8^+$ IELs) having a significant difference in the intercept only. Figure 3.34b illustrates that for $CD8^+$ IELs the cell numbers did remain elevated in comparison to the control values, but that the numbers remained relatively constant (as did the control values), and so there was no significance within the changing degrees of cellular infiltration between the two groups.

No significance could be attributed to the levels of $CD103^+$ or $\gamma\delta$ TCR^+ cellular infiltration, and Figures 3.28b and 3.30b illustrate the similarities in both cell numbers and the absence of changes in the degree of infiltration between inoculated and control values over time.

Spearman rank correlation analyses were employed to determine whether significance could be attributed to an association between the degree of cellular infiltration, and the number of crypts infected with *L. intracellularis*. The p values following these tests are presented in Table 3.5 below, and the associated graphs are in Appendix III. *L. intracellularis* was detected at day 14 only in WT mice and so this is the only time point included.

	$IFN\gamma R^{-/-}$			WT
	14	21	28	14
CD11b	0.152	0.881	0.932	1.00
CD3	**0.028**	0.214	0.606	0.147
CD3 IEL	**0.001**	0.425	0.205	0.347
CD4	**0.026**	0.234	0.606	0.768
CD8	0.738	0.365	0.765	0.300
CD8 IEL	0.803	0.803	0.283	0.792
CD103	0.841	0.676	0.232	0.983
$\gamma\delta$ TCR	0.662	0.276	0.637	0.897
plasma cells	0.596	0.321	0.200	0.104

Table 3.5. P values for Spearman rank correlation analyses. Significant p values (<0.05) are shown in bold.

The data demonstrated that in the majority of cases the degree of cellular infiltration (detected immunohistochemically) was not associated significantly with the proportion of crypts infected with *L. intracellularis* (Appendix III).

The significance attributed to the $CD3^+$ and $CD4^+$ populations at day 14 in the inoculated $IFN\gamma R^{-/-}$ group was removed when the data for those animals negative for the presence of *L. intracellularis* was no longer included.

3.4 Serological Responses.

Western blotting.

To analyse the serological response generated against *L. intracellularis* following challenge, plasma samples collected from WT and $IFN\gamma R^{-/-}$ mice (Experiment three, section 2.2.1.4) were blotted against *L. intracellularis* proteins separated on a polyacrylamide gel. Figure 3.44 is a representative illustration demonstrating antigenic components at a variety of molecular weights (as determined by a wide range Sigma marker). It illustrates the response seen in mice from Experiment three at days 21 and 28 post-inoculation. Both show the monoclonal antibodies sole recognition of a 24 kDa antigen of *L. intracellularis*. Recognition of the 25-27 kDa doublet by the plasma samples was taken as a demonstration of positive recognition of *L intracellularis* by the challenged animals. Western blot analysis showed that all animals inoculated with *L. intracellularis* recognised the same immunodominant antigens of 25-27 kDa. The antibody was seen at the first time point analysed (day 14), and remained until day 35 (even in the WT challenged mice that did not show signs of pathology at necropsy). The control animals showed no signs of recognition of *L. intracellularis* antigens at any time point.

Seroconversion was seen as a direct result of inoculation with *L. intracellularis* and was not apparent in control mice, at any point. The antibody response was specific for the 25-27 kDa immunodominant antigens and was not dependent upon the continued presence of bacteria or disease pathology in the gastrointestinal tract over the time period studied.

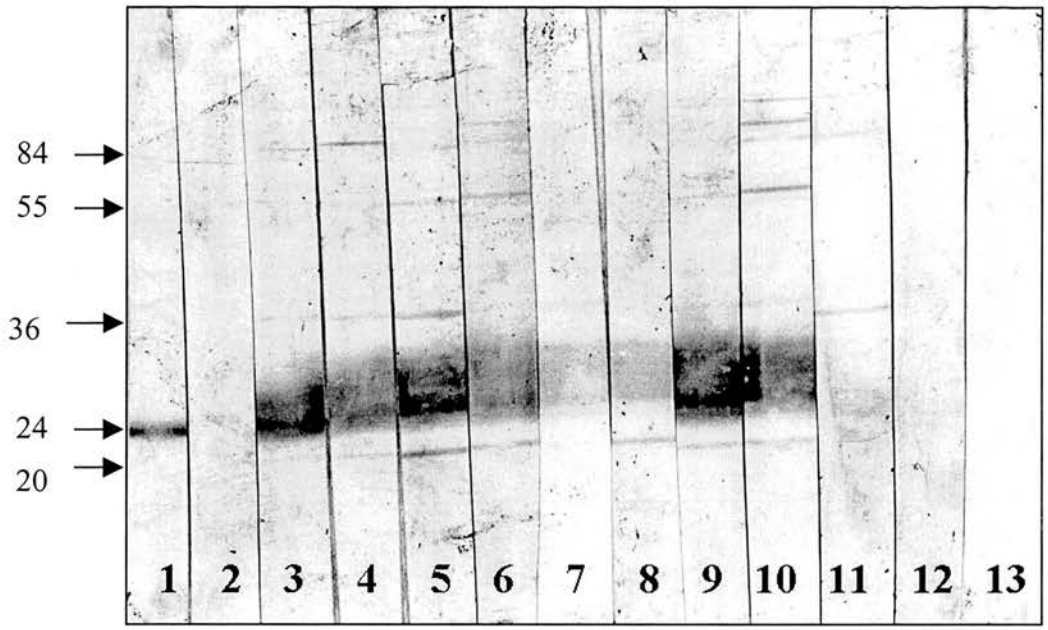


Figure 3.44a.

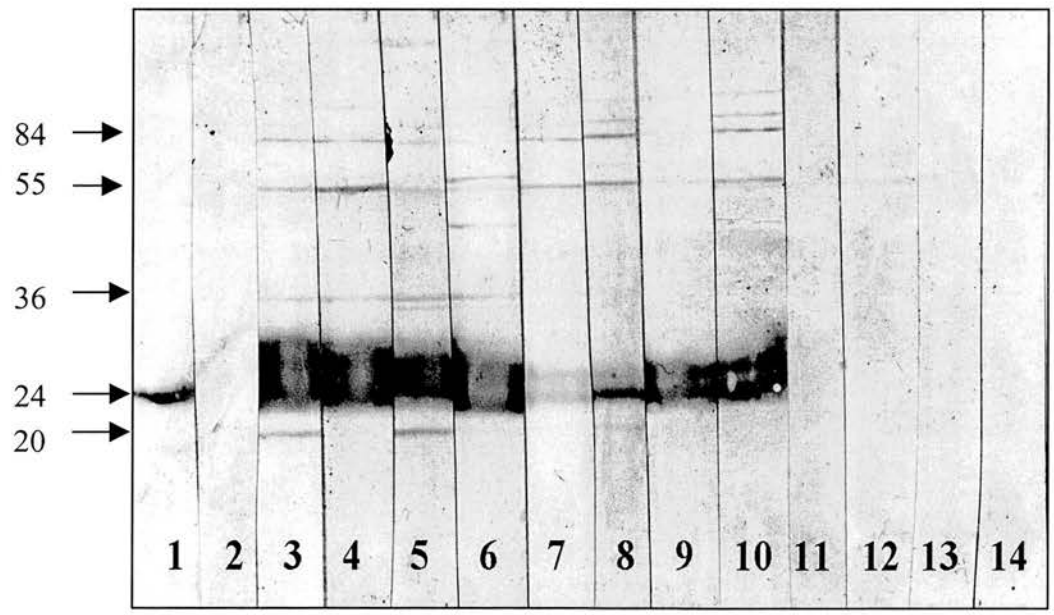


Figure 3.44b.

Figure 3.44. Western blot analysis following *L. intracellularis* separation on a polyacrylamide gel.
Incubated with dilutions of mouse plasma (1/100) from day 21 (a) and day 28 (b) post-inoculation.
Lane1, mouse monoclonal antibody (VPM53); 2, negative control; 3-6, WT mice inoculated with *L. intracellularis;* 7-10, IFNγR$^{-/-}$ mice inoculated with *L. intracellularis;* 11-12, WT control; and 13 (and 14 for day 28), IFNγR$^{-/-}$ control mice.
The secondary antibody was a rabbit anti-mouse HRP conjugate.
Weights as illustrated by arrows are in kilodaltons.

4. Discussion.

Lawsonia intracellularis is an obligate intracellular pathogen and the causative agent of proliferative enteropathy. It was classified as the sole cause of this enteric condition in 1993 (McOrist et al., 1993), but since then very little information has been determined regarding the pathogenesis and immune response generated against this novel bacterium. *L. intracellularis* is a unique organism with infection characterised by the hyperplasia of infected crypt enterocytes in the ileum and colon. The absence of detailed information regarding the immune response generated by the host in response to infection is further complicated by the general perceived absence of any inflammatory infiltration into the gut mucosa. This study aimed to establish an infection system in the mouse for *L. intracellularis* to enable further characterisation of the immune response against the intracellular bacteria. To aid in this characterisation IFNγR$^{-/-}$ mice, as well as conventional WT 129/Sv/Ev mice were incorporated into the study. The advantage of studying the infectious and immune processes in mice deficient in a functional IFNγR centres around the vital role of IFNγ in generating a protective immune response through its role in both the innate and specific arms of the response. For example in the activation of macrophages and iNOS, as well as the up-regulation of MHC class II antigen presentation, and cytotoxic T lymphocyte activation. All of these factors, and more, would be expected to play a vital role in the hosts protective response against an intracellular bacterium such as *L. intracellularis.*

4.1 Experimental rationale and development.

This work has provided the first demonstration of a reproducible infection system in 129/Sv/Ev wild type and IFNγR$^{-/-}$ mice, where lesions of proliferative enteropathy developed following inoculation with *L. intracellularis* and the immune response was studied over several weeks. Infection was determined immunohistochemically by the intracellular presence of *L. intracellularis*, and was associated with hyperplasia of infected enterocytes, and the loss of associated goblet cells.

The establishment of the model infection system also permitted assessment of the immune response following inoculation in both wild type mice and those deficient in the interferon gamma receptor. The infection system was achieved using wildtype (129/Sv/Ev) and isogenic gamma interferon receptor knockout (IFN$\gamma R^{-/-}$) mice. The animals were dosed orally with a suspension containing pure *L. intracellularis,* to represent the natural route of infection. At various time points post-inoculation they were euthanased, and sections of the ileum and colon examined for the intracellular bacteria. Histopathological analysis was performed on sections of colon from inoculated and control mice to look at the extent of induced inflammation and cellular infiltration. This was followed by a more specific analysis of the infiltration of T cells (immunohistochemical detection of $CD4^+$ and $CD8^+$ cells) in an initial study to begin characterisation of the immune response following infection with *L. intracellularis.* This pilot data permitted progress in protocol development (as detailed below), thus leading to more detailed statistical analysis using immunohistochemistry to detect $CD3^+$, $CD4^+$, $CD8^+$, $CD11b^+$ and $\gamma\delta$ TCR^+ infiltrating cells, as well as $CD103^+$ cells and its ligand E-cadherin in a later study.

The serological response against immunogenic antigens of *L. intracellularis* was also evaluated. Results are presented from Experiment three as the assessment at a single time point (immediately prior to post-mortem). The final challenge study aimed to incorporate serial analyses at weekly intervals throughout the period of study, beginning prior to inoculation with *L. intracellularis*, and this work remains in progress.

The establishment of a successful mouse infection system has resulted in reproduction of lesions characteristic of proliferative enteropathy (PE) following infection with *Lawsonia intracellularis*, and constituted a major part of this study. These defining experiments secured an animal model that was both representative of the natural disease, and useful in its ability to dissect the pathogenesis of the organism using genetically manipulated hosts.

The responses seen in wildtype mice following inoculation with *L. intracellularis* (that appeared capable of either eliminating or resisting infection), could be compared with those seen in isogenic mice lacking effective IFNγ signalling, where immunity against *L. intracellularis* was compromised.

L. intracellularis is a highly fastidious obligate intracellular organism and consideration must be given to the difficulty in growing sufficient organism *in vitro* for *in vivo* challenge experiments. It is therefore difficult to obtain sufficient inoculum for large scale experiments. For instance 4-6 weeks is required to obtain a bacterial suspension of approximately 10^8 organisms.ml^{-1}. In the experimental protocol used this was sufficient for approximately 16 animals. Despite this difficulty sufficient inoculum was produced for animal groups of greater numbers as studies progressed.

Previous examples of proliferative enteropathy in the mouse were restricted to those reproduced following inoculation with swine PE intestinal homogenate (Chang et al., 1985) rather than a pure culture of *L. intracellularis.* Collins et al., (1999) inoculated several strains of mice with *L. intracellularis*, but less than 10% of animals inoculated developed lesions of PE. Furthermore the experiment contained insufficient groups, or sufficient numbers of mice within these groups to allow either statistical analysis or the opportunity to explore pathogenic and immune mechanisms.

Natural outbreaks of proliferative enteropathy occur in an ever increasing range of host species, probably due to increased awareness of the pathogen and its pathology. Challenge studies with cultures of *L. intracellularis* have also been documented in a variety of animals including pigs (McOrist et al., 1993), hamsters (Jasni et al., 1994 a and b) and rats (Collins et al., 1999). For the purposes of establishing a mouse infection system for proliferative enteropathy 129/Sv/Ev WT and isogenic interferon gamma receptor knockout (IFNγR$^{-/-}$) mice were challenged orally with a suspension of *L. intracellularis* grown in cell culture. Inoculation was via gavage, and mimicked the natural route of infection, with the inocula being delivered directly to the stomach.

The numbers of mice in each inoculated and control group varied as studies progressed and are given in Table 2.2 (Materials and Methods). The first three experiments had groups of four mice inoculated with *L. intracellularis*, and control groups of 2 at the designated time points. Variations between the experimental design at this point included the exclusion of day 7 post-inoculation from all bar the initial study (due to the absence of any infection at this stage). Also analysis of 40 days post-inoculation was discontinued in preference for analysis of significant numbers at seven day intervals. Initial studies allowed progressive refinement with the aim of the final experiment being a more comprehensive analysis of the progressing immune response following inoculation with *L. intracellularis.* Based on previous experimental challenge studies the data demonstrated that at the peak incidence of infection at least 50% of inoculated wild-type animals had lesions of proliferative enteropathy therefore group sizes of both inoculated and control groups were increased for improved statistical power in the analysis of results.

A group size of 10 animals (to be inoculated with *L. intracellularis*) was therefore considered a requirement for generating a minimum of 5 mice which develop lesions of proliferative enteropathy. A minimum group size of 5 was used for all control groups of mice (inoculated with buffer only).

Assessment of infection (and immune cell infiltration) was carried out at 14, 21 and 28 days post-inoculation rather than at a single time point to follow progression of infection and immune response. Analysis of a single time point was not deemed to provide sufficient information based on previous experimental data. For example earlier results have shown that infection in wild-type mice inoculated with *L. intracellularis* reached their peak at 14 to 21 days post-inoculation (therefore suggesting both these dates are important for further study). Infection rates with *L. intracellularis* in IFNγR$^{-/-}$ mice appeared to be maximal by day 21 post-inoculation (and then remain high).

Hence studies incorporating both 14 and 21 days post-inoculation were an absolute requirement for this experiment to have the greatest likelihood of incorporating the peak time points of infection for both inoculated wildtype and IFNγR$^{-/-}$ mice. The study was then extended for a further 7 days to increase the strength of the data. In particular this was to assess the potential resolution of infection in the WT animals in comparison to continuing infection in the IFNγR$^{-/-}$ mice.

4.2 Clinical and gross pathological features associated with PE.

All experimental studies demonstrated that the control animals (given SPG buffer by gavage) remained consistently negative for the presence of *L. intracellularis,* or lesions of PE, following histopathological examination, as expected.

Proliferative enteropathy presents with a variety of different clinical manifestations in the pig (including anorexia and diarrhoea), and in many cases is thought to be sub-clinical (Lawson and Gebhart, 2000). Experimental studies however have indicated that weight loss is not a reliable clinical indicator of infection in either the hamster or the pig (Smith et al., 1998). In development of the infection system presented here one aim was to determine whether similar symptoms were associated with disease in the mouse.

The weights of all mice were monitored over the duration of each study. In the majority of cases there was no trend towards weight loss as a symptom of proliferative enteropathy. Figures 3.7 and 3.21 illustrate examples, however, where relative weights were calculated as either a positive or negative differential between the time of gavage (day 0) and post-mortem (day 14, 21, 28 or 35). Infected IFNγR$^{-/-}$ mice demonstrated a statistically significant decrease in the weight of animals over time (3.3.1), in comparison to the control animals that exhibited a weight gain, as would be expected.

The weight differentials over time between the control and inoculated WT mice in these experiments were not significantly different from each other. These results indicated that weight loss was not a consistent feature of proliferative enteropathy associated with the mouse model described in WT mice, but was a notable feature in the disease of IFNγR$^{-/-}$ mice.

Another symptom of PE seen in the pig is diarrhoea, and this was occasionally apparent in highly infected IFNγR$^{-/-}$ mice at later time points (days 28 or 35). At these later stages a number of IFNγR$^{-/-}$ mice were also failing to thrive and were less active than the control cohort group. In the majority of cases, however, mice (both WT and IFNγR$^{-/-}$) demonstrated lesions of PE at necropsy but did not display clinical symptoms or signs of illness throughout the study.

The diagnosis of proliferative enteropathy is most frequently made following post mortem examination where the gross and histopathological lesions in the gastrointestinal tract are characteristic. Gross lesions in the pig can present with thickening of the ileum and colon, with an apparent corrugated appearance, and in the case of the acute form (proliferative haemorrhagic enteropathy, PHE) blood clots can be seen in the lumen of the gastrointestinal tract (Lawson et al., 1979). Previous reported cases of proliferative enteropathy in either mouse or hamster experimental infections have not reported evidence of gross lesions (Collins et al., 1999 and Jasni et al., 1994a) whereas gross changes have been noted in both the pig and rabbit. In the rabbit changes are seen along the length of the gastrointestinal tract from the colon and ileum (Schauer et al., 1998) to the jejunum (Hotchkiss et al., 1996).

The initial challenge experiment with 129/Sv/Ev and IFNγR$^{-/-}$ mice (3.1.1A) did not demonstrate gross lesions in either the colon or ileum at any time point following challenge with *L. intracellularis* (despite the presence of characteristic PE lesions on microscopic examination).

In subsequent experiments, while no gross changes were detected in any of the control groups, a proportion of IFNγR$^{-/-}$ mice challenged with *L. intracellularis* demonstrated gross changes. This was apparent as visible thickening of small regions of the colon (with any changes observed in the ileum restricted to the final experiment) with a corrugated appearance (Figure 3.1).

While no significant gross thickening of the colon was seen in any inoculated WT or IFNγR$^{-/-}$ mice in the initial study there was evidence of macroscopic changes in the intestine of both WT and IFNγR$^{-/-}$ mice in the final three experiments. Similar gross changes were also a feature of two further challenge experiments with virulent *L. intracellularis*, data not shown. The changes observed in WT mice, however, were not as marked as those seen in the more heavily infected IFNγR$^{-/-}$ mice. Several IFNγR$^{-/-}$ mice in the final experiment also demonstrated slightly thickened regions of the ileum. Previous assessment of colonic weight as a marker for epithelial hyperplasia in proliferative enteropathy (of mice) has not proven suitable when comparing infected and control sections of tissue (data not shown). Although others have shown significant increase in colon weight during transmissable murine colonic hyperplasia (Higgins et al., 1999b) this did not prove to be a reliable marker in *L. intracellularis* infection of mice despite evident intestinal thickening and histological evidence of hyperplasia.

In a small number of inoculated IFNγR$^{-/-}$ animals in Experiment Two (see Figure 3.1) and Experiment Four, there was evidence of blood clots in the lumen of grossly (and microscopically) proliferative colons. This presentation resembled that associated with proliferative haemorrhagic enteropathy (PHE), the acute form of PE seen in the pig (Rowland and Lawson, 1975). The animals also demonstrated loose bloody faeces and were highly anorexic in comparison to their cohorts, and the control group.

These observations demonstrated that while gross thickening of the gut was not a typical pathological feature of PE seen in infected wild-type mice, it was an aspect associated with the more pronounced infection seen in IFNγR$^{-/-}$ mice. It also corroborated the close parallel between the natural disease in pigs, and the experimental model established here. The gross thickening of the gut seen is attributable to the increased epithelial cell proliferation as a result of infection with *L. intracellularis*. Rarely natural disease has also been reported to include enlargement of mesenteric lymph nodes (MLNs) with evident intracellular organisms (Roberts et al., 1980). Analysis of MLNs was assessed in the two preliminary challenge experiments, and while on occasion enlarged mesenteric lymph nodes were seen, there was no evidence of intracellular bacterial multiplication.

4.3 *L. intracellularis* – location.

In the pig infection with *L. intracellularis* is associated with loss of villi in the ileum (Lawson and McOrist, 1993). In the mouse model, infection was consistently more pronounced in the colon in comparison to the ileum. Similar features noted, however, were the increased crypt lengths, and increased level of epithelial renewal.

L. intracellularis was detected in the apical cytoplasm of proliferating enterocytes, a feature of proliferative enteropathy that has been established across a variety of host species. The proportion of crypts infected with *L. intracellularis* was determined by IHC since direct microbiological quantitation of the organism by routine colony counts was not possible due to the obligate intracellular nature of the bacterium and the difficulty in cultivation. Immunohistochemical detection of *L. intracellularis* proved highly sensitive with levels of infection as low as 1 crypt in 300 clearly detectable. Alternative means of detection include the non-specific Warthin-Starry silver stain, and the polymerase chain reaction (PCR). While the former is most frequently used in widespread clinical diagnosis, the latter, along with immunohistochemical analysis, are preferable due to their specificity for *L. intracellularis*.

Various studies have evaluated the differences between PCR and immunohistochemistry (IHC) as the most efficient and sensitive way to detect *L. intracellularis,* with conflicting results. Jordan et al. (1999) demonstrated an increased sensitivity with PCR detection of the organism from infected mucosa. However, MacIntyre (2001) found that there was no advantage in detection by PCR, in comparison to IHC. Immunohistochemical detection of *L. intracellularis* is a valuable post-mortem test, and the advantages over the alternative PCR methodology include the fact that it is less time-consuming, laborious and expensive (as well as providing additional histological details) and therefore more practical for routine diagnosis. An alternative ante-mortem test for *L. intracellularis* has been tested using PCR analysis of faecal samples from affected pigs. This, however, has been shown to be limited to animals with active lesions (McOrist et al., 1994b), therefore suggesting the possibility of false negatives when animals infected but not shedding the organisms are tested (Knittel et al., 1997).

L. intracellularis is an obligate intracellular pathogen, and as such has an absolute requirement for a susceptible host cell for its continued replication and survival. It multiplies within the rapidly dividing immature crypt epithelial cells of the gastrointestinal tract, principally the ileum and jejunum of pigs (Lomax and Glock, 1982) although it is also seen in the colon. There has been some evidence that the principal site of multiplication can vary depending upon the host species infected. For example in hamsters lesions are seen in the ileum and jejunum (Jasni et al., 1994a). Horse, deer and ostrich demonstrated lesions in the ileum (Cooper et al., 1997a), while rabbits have shown lesions in the ileum, colon (Schauer et al., 1998) and jejunum (Hotchkiss et al., 1996). In macaques infection was only detected within the ileum (Klein et al., 1999). Infection in the reported mouse model indicated an apparent predilection for a more pronounced infection in the colon of 129/Sv/Ev WT and IFN$\gamma R^{-/-}$ mice compared to the ileum (with an increased number of enterocytes infected with *L. intracellularis*, and a higher degree of associated epithelial hyperplasia). Infection and associated pathology were, however, still seen in both the ileum and colon.

Histopathologic analysis revealed that the intestinal distribution of *L. intracellularis* was not affected by the IFNγR deficiency. However, the mutation allowed the unrestricted multiplication of the organism in the intestine, and in contrast to wt mice IFNγR$^{-/-}$ mice appeared unable to eradicate the parasite from infected areas.

L. intracellularis has been confirmed as the cause of rectal prolapse in emus (Lemarchand et al., 1997), where the distal rectal mucosae presented with typical pathological findings associated with proliferative enteropathy (this included grossly thickened mucosae, histologically evident enterocyte hyperplasia, decreased goblet cell density and intracellular *L. intracellularis*). Although the large intestine was most severely affected, inflammation and organisms were also present in the small intestine. Infection demonstrated *L. intracellularis* within the apical cytoplasm of enterocytes in the rectum from the bottom of crypts to the tips of villi. This finding is notable in that disease in mammals has previously been restricted to proliferation of *L. intracellularis* within the immature epithelial cells found in the crypts, and not in the more differentiated cells found in the villi and epithelium of the gut. The infection system presented here in mice, however, is another exception to the accepted dogma since *L. intracellularis* was detected both within the crypt enterocytes and the mucosal epithelium lining the folds of the surface of the colon in infected 129/Sv/Ev IFNγR$^{-/-}$ mice (Figure 3.6) but not in WT animals. The maturation state of these infected cells has not been ascertained but would be an interesting avenue to pursue. For example do they possess markers of continued proliferation such as Ki-67 or c-myc (normally associated with the highly proliferative crypts rather than mature epithelial cells) which may indicate an altered differentiation state. If this were the case the ensuing question would centre on the cause and effect pathway regarding infection with *L. intracellularis*. For example *L. intracellularis* has previously been shown to demonstrate a strong tropism for rapidly dividing cells, both *in vitro* and *in vivo*.

Whether *L. intracellularis* displays the ability to enter and survive within mature epithelial cells, or has the ability to alter the differentiation state of the epithelial cells that it infects, (so that they remain immature, but still follow the normal pathway of epithelial cell regeneration from progenitor cells), is not known, but would be interesting to investigate further.

Electron microscopy has contributed towards the continuing study of the interactions between intracellular pathogens and their host cells, and was used here to further investigate the tropism of *L. intracellularis*. Previously, lesions of PE have demonstrated the presence of intracellular organisms correlated with the proliferation of crypt enterocytes, to the exclusion of mature villous cells (Rowland and Lawson, 1975). In this study organisms morphologically consistent with *L. intracellularis* were present within immature enterocytes of the colon of infected IFN$\gamma R^{-/-}$ mice (Figure 3.4) and were also seen within epithelial cells of the mucosal folds of the colon (Figure 3.6). These organisms were clearly seen within the apical cytoplasm of the cells, and were non-membrane-bound as seen in natural disease in the pig. The maturation state of the cells was not assessed but stunted microvilli were present suggesting some aspects of cell differentiation may have been altered. It is still not known however, if the infected cell is a mature cell, or an immature cell that has migrated through the epithelium due to altered cell dynamics following infection. There was a high correlation between the organisms seen following electron microscopy and *L. intracellularis* detected immunohistochemically, both morphologically and anatomically (as both were found at the apical cytoplasm).

4.4 *L. intracellularis* and epithelial hyperplasia.

Following inoculation with *L. intracellularis* the infection remains focal, with a sharp contrast between adjacent areas of healthy and infected tissue (perhaps reflecting mode of replication and cell-cell spread of the organism, Lawson et al., 1995).

An associated loss of goblet cells was assumed to result from the inability of these enterocytes to mature and further differentiate into the effector cells required for normal intestinal function. This is the classic presentation of proliferative enteropathy seen in a variety of species (Lawson and Gebhart, 2000). Similar intestinal changes including increased enterocyte proliferation and depletion of goblet cells can be seen in a variety of mice, including T cell receptor (TCR) mutants, which develop spontaneous inflammatory bowel disease (Mombaerts et al., 1993b), therefore suggesting involvement of the immune response in the development of hyperplasia.

Epithelial hyperplasia is a recognised pathological feature associated with *L. intracellularis* infection. Infection of the rapidly dividing immature crypt cells of the ileum and colon results in focal lesions of hyperplastic crypts, adjacent to areas of healthy uninfected epithelium. These hyperplastic lesions clearly present with an increase in the number of cells per infected crypt and this is seen in all species that have presented with lesions of proliferative enteropathy. Of particular interest is the fact that the mouse model discussed here presents with more pronounced hyperplasia in infected IFN$\gamma R^{-/-}$ mice than their wild-type counterparts, with significantly more enterocytes per infected crypt ($p<0.05$). Analysis of the sections of infected tissue also illustrated an increase in mitotic figures in these hyperplastic crypts. Further studies in a pig experimental challenge (MacIntyre, 2001) demonstrated that crypt cell proliferation in infected intestinal enterocytes was increased significantly and this was associated with the continued presence of *L. intracellularis.* Cell changes were assessed by evaluating the expression of Ki-67 (a nuclear non-histone protein which is a good marker of proliferation associated with all actively dividing cells), as well as mismatch repair protein (MMRP), and the oncogenes bcl-2 and c-myc. Combined, these analyses demonstrated that infected crypts were characterised by up-regulation of oncogenes with increased cell proliferation, and inhibition of apoptosis. These changes suggest that the enterocytes were actively involved in a pathway of increased proliferation, rather than the accumulation of crypt cells resulting from a lack of differentiation of these immature progenitor cells.

Further studies will be required to determine in more detail the methods of dysregulation induced by *L. intracellularis* infection and the changes in markers of epithelial cell proliferation in the murine infection system.

What remains to be elucidated is whether the proliferation of epithelial cells in IFNγR$^{-/-}$ mice is increased directly due to the absence of functional IFNγ, indirectly through the disruption of regulatory cytokine cascades or if uncontrolled growth of *L. intracellularis* is responsible for increased proliferation. It is interesting in this respect to compare and contrast the immunological and proliferative aspects of two other intestinal pathogens, *Citrobacter rodentium* and *Trichuris muris*. *C. rodentium* provides a contrasting example of dysregulated cell proliferation to that seen following infection with *L. intracellularis*. The disparity between the two infections results as the hyperplasia induced in WT animals following infection with *C. rodentium* is absent in inoculated IFNγR$^{-/-}$ mice (Higgins et al., 1999b), whereas infection with *L. intracellularis* induces hyperplasia in both backgrounds of mice. Infection with *C. rodentium* also resulted in crypt hyperplasia, epithelial cell proliferation and mucosal thickening (Higgins et al., 1999a). Proliferation was due to a Th1 response, via intimin, and thereby initiated by the organism and not the host. In the absence of a functional IFNγ receptor the response to infection with *C. rodentium* was varied noticeably. This included a decrease in the presence of TNFα, IFNγ and KGF transcripts in comparison to infected WT animals (where IL-4 was also decreased). The lamina propria T cell infiltration was predominantly CD4^{+}, as it is following infection with *L. intracellularis*. Intimin is necessary to induce inflammation and crypt hyperplasia (as well as acting as a ligand for epithelial cell adhesion to Tir in the formation of the attaching and effacing lesion).

A further contrasting example can be seen following *Trichuris muris* infection, an intestinal nematode (Artis et al., 1999). Chronic infection with the pathogen is associated with an inappropriate Th1 cytokine response and the increased production of IFNγ.

Consequently the removal of IFNγ from an *in vivo* infection system ameliorates the hyperproliferation associated with disease. Therefore illustrating another situation in which IFNγ was involved in the regulation of epithelial cell proliferation, although it apparently did not have a regulatory role in the noninflamed gut (Artis et al., 1999). While the absence of a functional IFNγ pathway clearly does not remove the hyperplasia during *L. intracellularis* infection, it is clear that the absence of the cytokine receptor results in the dysregulation of other pathways co-ordinating both epithelial proliferation and T cell mediate immunity.

Increased epithelial proliferation as a result of infection with *L. intracellularis,* however, was not dependent on the presence of a functional IFNγ signalling pathway, and presumably utilises a distinct pathway that results in the characteristic disease pathology. MacDonald (1992) reports that increased proliferation of the epithelium may be associated with no T cell activation therefore suggesting that hyperplasia may occur in response to different inflammatory stimuli. Alterations in cytokines such as keratinocyte growth factor (KGF) and matrix metalloproteinase (MMP) production by intestinal myofibroblasts can also affect intestinal mucosal shape and integrity. The production of KGF by myofibroblasts is upregulated by cytokines (Chedid et al., 1994) and then acts on epithelial cells via its receptor (Miki et al., 1991) to promote epithelial growth. Myofibroblasts increase their level of KGF production *in vitro* following TNFα activation, but are unresponsive to IFNγ (MacDonald et al., 1999). Experiments have shown, however, that TNFα is also capable of inducing crypt cell hyperplasia directly and that KGF activates epithelial cells to make TNFα, which then acts in an autocrine manner to increase crypt cell division. Intestinal myofibroblasts are the main producers of KGF, although evidence also exists for the production of KGF by γδ TCR^+ IELs in mice (Boismenu and Havran, 1994).

Lesions characteristic of proliferative enteropathy presented with exclusive hyperplasia of immature crypt cells infected with *L. intracellularis.* The focal nature of infection with *L. intracellularis,* and the associated pathology, is in marked contrast with adjacent areas of healthy epithelium. This may arise as a consequence of the continued division of infected epithelial cells, with the resultant daughter cells containing dividing organisms (Lawson et al., 1993). There is also evidence of this phenomenon *in vitro* (Collins et al., 1996). Hyperplasia is only seen in crypts infected with *L. intracellularis*, suggesting that infection with the organism may be a requirement for increased epithelial proliferation. A higher proportion of crypts infected with *L. intracellularis* (and the number of enterocytes per crypt) was evident in diseased IFN$\gamma R^{-/-}$, in comparison to wildtype, mice.

As can be seen in all these examples of gastrointestinal disease (*L. intracellularis, C. rodentium, T. muris*) a delicate balance is required for the control of epithelial cell growth and differentiation, as well as immunoregulation in the gastrointestinal tract. This can be highlighted by the development of spontaneous colitis in mice deficient for certain elements important in regulation of the immune system. For example IL-2 (Sadlack et al., 1993), IL-10 (Kuhn et al., 1993) and TCR (Mombaerts et al., 1993b) deficient mice all develop enterocolitis as a consequence of the imbalance generated in intestinal immunoregulation. Intestinal pathology resembles inflammatory bowel disease and consists of mucosal thickening, crypt hyperplasia, goblet cell loss and general inflammation of the lamina propria. Inactivation of the IL-2 gene induces inflammatory responses leading to IBD either directly or indirectly through the complex deregulation resulting from the IL-2 deficiency. The animals have a high number of activated T and B lymphocytes in the colonic mucosa, a relative reduction in $\gamma\delta$ TCR^{+} cells, and elevated IgG1 and autoantibody expression (Sadlack et al., 1993). A later study (Ma et al., 1995) demonstrated that disease developed in immunoglobulin deficient mice, indicating the colitis was not antibody-mediated as first thought, and also indicating a primary role for T cells.

It is interesting that IBD develops in the colon and caecum of IL-$2^{-/-}$ mice, while the ileum appears normal with only minor changes in cell infiltration and cytokine mRNA, suggesting a subtle immune response (McDonald et al., 1997). Differences between the ileum and colon in these mice would be consistent with the high degree of functional and phenotypic specialisation among T cells of the gastrointestinal tract. This includes phenotypic variations between the ileum and colon of mice, and variations between different strains (Beagley et al., 1995). A crucial role for the resident bacterial flora is evident in IL-2 (Sadlack et al., 1993) and IL-10 (Kuhn et al., 1993) deficient mice, with inflammation absent in germ-free or specific pathogen-free animals. Alterations in the equilibrium within the gastrointestinal tract clearly have a 'knock-on' effect resulting in inflammation and pathological changes as a consequence of an aberrant Th1 response directed at intestinal antigens. IL-10 is not needed for the differentiation of Th2 cells, but does inhibit the development of Th1 cells during a Th2 response *in vivo* (Kuhn et al., 1993), and therefore has an essential role in controlling intestinal immune responses directed to enteric antigens. As with all altered responses following genetic manipulation of the host it remains an open question whether the differences reflect a true compartmentalisation of function; whether there is insufficient compensation in the particular organ being examined; or if the changes result indirectly due to complex deregulation as a result of the deficiency i.e. IL-2 or IL-10.

Lesions of proliferative enteropathy were diagnosed in inoculated mice following immunohistochemical staining of sections of the ileum and colon (Lawson et al., 1985; McOrist et al., 1987). Infected animals presented with bacteria evident principally in the apical cytoplasm of immature enterocytes. These crypt epithelial cells demonstrated an increased rate of proliferation, a feature not seen in adjacent uninfected crypts.

All challenge experiment data consistently indicated that inoculated IFN$\gamma R^{-/-}$ mice demonstrated a higher incidence of infection than wild-type animals (Table 3.3). In general there was an equal, or higher, incidence of infection in challenged IFN$\gamma R^{-/-}$ mice than in isogenic WT animals the only exception being day 14 in Experiment One.

This implies that the IFNγR plays a vital role in the protective response of the immunocompetent host against the infection with this organism, and one which cannot be fully compensated for by the remaining functional aspects of the immune system.
WT mice appeared to either resist initial infection or develop resistance to disease (129/Sv/Ev) with the ability to eliminate infection with *L. intracellularis* over the time period studied. The assumption was that the mice developed a protective response to infection with *L. intracellularis,* and that disease was established early on in all WT animals, but was only detected at 14 and 21 days post-inoculation due to elimination of the infection. This belief is supported by the cell infiltration patterns and alterations in intestinal pathology. For example in the final experiment, day 14 presented with the highest number of infiltrating cells for all phenotypes, as well as the highest number of enterocytes per crypt, and the lowest number of goblet cells. Subsequently these values returned to normality by day 28. Perhaps the most telling point in this regard is that at day 21 the cells were midway between these two points, suggesting that they were previously active but their role was now completed. As it is not possible to monitor infection in individual mice over time, statistical analysis grouped together all inoculated animals, rather than separating diseased and non-diseased, and compared these with control values. It is also not known at any point whether the proportion of infected crypts is increasing or decreasing, or whether an animal inoculated with *L. intracellularis*, but with no evidence of infection managed to clear an early infection or resist establishment of disease.

Infection in the IFNγR$^{-/-}$ mice in Experiments Two and Three showed signs of a potentially resolving infection, with the percentage of infected crypts at day 35 lower than that for the preceding time point. In both of these experiments several mice died due to the development of severe lesions associated with large numbers of *L. intracellularis* prior to day 35. A logical interpretation of these findings would be that if an animal survives past this apparent crucial point, it may then go on to resolve the infection.

This scenario would parallel that seen in the pig, where infection is thought to peak at 21 days post-infection, and resolve naturally over a period of 6 weeks (Rowland and Lawson, 1992). However, *L. intracellularis* remained detectable within the gastrointestinal tract of all inoculated IFNγR$^{-/-}$ mice from day 21 in the final two experiments.

4.5 Host response to *L. intracellularis*.

Intracellular bacteria multiply within a protected niche of the host, but are exposed to the defences of the parasitised host cell. This is particularly significant in the case of infected macrophages, which can be stimulated to a bactericidal state by IFNγ. *L. intracellularis,* however, appears to multiply exclusively within non-phagocytic epithelial cells (with, to date, no strong evidence for growth in macrophages *in vivo*). Epithelial cells, although not directly bactericidal, may still play an important part in the developing immune response due to their potential ability to present peptides in association with major histocompatibility antigens (Mayer, 1997) and both their secretion and response to cytokines. The loss of a functional interferon gamma receptor clearly has an inhibitory effect on the ability of the knockout mice to eliminate infection with *L. intracellularis*, with infection persisting for a greater length of time, and reaching a more pronounced level. Studies on control versus IFNγR$^{-/-}$ mice have shown that there are apparently no differences in the expression of CD3, CD4, CD8, cell surface IgM or MHC I/II molecules in IFNγR$^{-/-}$ mice in comparison to WT mice (Huang et al., 1993). Factors of the immune system that were modified in these mice included NK cell stimulation and total serum IgG2a in addition it was shown that IFNγ also regulated the basic microbicidal activities of macrophages and neutrophils.

There was an obvious and detrimental difference between infection in the WT and IFNγR$^{-/-}$ mice following an equivalent dose of *L. intracellularis*. To elucidate the resultant modified immune response in the mutant mice a variety of aspects of both the innate and specific immune system were assessed.

Histopathology.

Although the recruitment of inflammatory cells is thought not to be typical of PE in swine (McOrist et al., 1992) it has been reported both in pigs (Lomax and Glock, 1982), and a variety of other animals (including macaques, Klein et al., 1999; emus, Lemarchand et al., 1997; and rabbits, Hotchkiss et al., 1996). Lomax and Glock (1982) reported an infiltration of eosinophils, neutrophils and macrophages in the lamina propria adjacent to infected crypts (following assessment of naturally occurring porcine PE). They further commented on the presence of similar intracellular organisms within the phagolysosomes of macrophages. Knittel et al. (1997) also reported the association of an inflammatory exudate and increase in mononuclear cells in the lamina propria in association with PE. Currently, however, no comprehensive study has examined, in a structured manner, the changing immune response over a time course of disease progression.

The cellular response following infection of WT and IFNγR$^{-/-}$ mice with *L. intracellularis* was analysed by routine histopathology over a minimum period of 28 days (Experiment three, Figures 3.8-3.15; Experiment four, Appendix II). The infiltration was assessed in comparison to the normal resident population of cells seen in the control animals. This demonstrated a mixed inflammatory response comprising neutrophils, macrophages and lymphocytes in both WT and IFNγR$^{-/-}$ mice inoculated with *L. intracellularis*. The response persisted in the knockout mice until the final time point studied (either 28 or 35 days post-inoculation) and this was presumably due to the ongoing infection with *L. intracellularis* in the IFNγR$^{-/-}$. In contrast, there was no demonstrable presence of *L. intracellularis* after 21 days post-inoculation in WT mice (Table 4.1, Experiment three) and this coincided with the cellular infiltration in the lamina propria returning to normal resting levels by 28 days post-inoculation. Other examples of an inflammatory response to *L. intracellularis* are seen in emus, with the villi of the rectum markedly broader and thicker due to the infiltration of plasma cells, macrophages, lymphocytes and heterophils (Lemarchand et al., 1997).

The response in the rabbit also exhibits increased numbers of lymphocytes, macrophages and heterophils (Schoeb and Fox, 1990). It is apparent that the response to *L. intracellularis* is variable among species, but there is evidence for a more pronounced inflammatory response in other species than has previously been documented in the pig (McOrist et al., 1993).

The manifestation of marked cellular infiltration following challenge with *L. intracellularis* in both infected WT and IFNγR$^{-/-}$ mice clearly illustrated that a functioning IFNγ signalling pathway was not required to induce an influx of inflammatory cells. Deckert-Schluter et al. (1996) demonstrated that IFNγR$^{-/-}$ mice responded to infection with *Toxoplasma gondii* in a similar way to inoculated WT mice, with the activation of CD4^{+} and CD8^{+} T cells, and recruitment of inflammatory cells to the area of inflammation. This aspect of the response was apparently independent of IFNγR expression yet the IFNγR$^{-/-}$ mice were more susceptible to toxoplasmosis than WT mice. This was shown to be as a result of the inefficient activation of recruited macrophages. The absence of a functional IFNγR resulted in reduced MHC II expression on macrophages (in comparison to WT mice), and was in conjunction with reduced activation of these cells (e.g. decreased production of TNFα, inducible nitric oxide synthase, and IL-1β). The reduced expression of MHC class II antigens was not likely to be responsible for defective innate immunity, but may have had a negative influence on specific immunity. Further support for the requirement of IFNγ activated macrophages in protection against toxoplasmosis came from an adoptive transfer study where immune T cells from WT animals did not protect IFNγR$^{-/-}$ mice and were obviously not able to compensate for loss of the innate function.

Initial histopathological analysis demonstrated an evident inflammatory response in mice inoculated with *L. intracellularis*. To construct a more detailed picture of the immune response following infection specific immunophenotyping studies were carried out to ascertain the relevant infiltrating cell populations.

Immunophenotyping the response to *L. intracellularis.*

Further evaluation of the innate immune response following inoculation with *L. intracellularis* was obtained by the immunohistochemical detection of the $CD11b^+$ cell population in the lamina propria of the colon. Mac-1 (CD11b/CD18, complement receptor 3, CR3) is a β2 integrin expressed on leukocytes and is expressed on neutrophils, monocytes/macrophages, NK cells, eosinophils and basophils and mediates adhesion to the endothelium. Assessment of the $CD11b^+$ population permits a broad spectrum view of a variety of cells, principally of the innate immune system, which may be involved in the elimination of infection with *Lawsonia intracellularis.* Investigation of the role of NK cells both as secretors of IFNγ, and as responders to increased levels of IL-12 would be relevant. Additionally, macrophages are specifically of interest due to their involvement in the elimination of bacterial infection, as well as their altered activation states in $IFN\gamma R^{-/-}$ mice (as mentioned previously in toxoplasmosis). While immunohistochemical detection of $CD11b^+$ cells allowed a general overview of the situation clearly, in these circumstances, immunostaining for a specific macrophage marker would be advantageous. While this was attempted on both frozen and fixed samples of tissue collected in this study, using a variety of potential markers, it was unfortunately unsuccessful in generating reliable positive staining in the sections of colon analysed. There was an evident increase in the number of infiltrating $CD11b^+$ cells in both WT and $IFN\gamma R^{-/-}$ mice inoculated with *L. intracellularis.* The cell numbers remained high in the lamina propria of $IFN\gamma R^{-/-}$ mice, concomitantly with the presence of *L. intracellularis.* The statistically significant changes in the cell population (slope, $p=0.021$) seen in the WT mice, but not evident in the $IFN\gamma R^{-/-}$ mice ($p=0.433$) is indicative of the initial increase in cell infiltration at day 14 which then returns towards control values as the pathogenic stimulus is removed. This demonstrates that the absence of the IFNγR does not appear to have a marked affect on the infiltration of the $CD11b^+$ population, the consequences on cell activation, however, were not determined. This aspect is one where the loss of a functional IFNγ signalling pathway may be of extreme importance, as detailed below, and one worthy of further investigation.

For example assessing the variance in iNOS production in the two backgrounds of mice following inoculation with *L. intracellularis,* as well as the cytokine milieu affected by disruption of the IFNγ signalling cascade and subsequent development of Th1/Th2 responses.

Although a significant aspect of the immune system appears unaltered by the absence of a functional IFNγ signalling pathway with infiltration of inflammatory cells and normal T and B cells (Huang et al., 1993), either because the cytokine is not directly required, or its functions are compensated for, there are some aspects of the immune response affected by its absence. These include macrophages and natural killer (NK) cells that demonstrate a requirement for IFNγ. Dai et al. (1997) demonstrated that IFNγR$^{-/-}$ mice displayed defective innate immunity following challenge with *L. monocytogenes* attributable to the altered function of macrophages and the lack of production of iNOS by IFNγR$^{-/-}$ mice. They also illustrated that the recruitment of phagocytic cells to infected cells was maintained (and dominated by neutrophils which contributed to the inflammatory cytokine response by their expression of TNFα and IL-1α). These cells, however, were unable to fully protect the host from infection. NK cells also have an altered function, with reduced stimulation due to the absence of the IFNγR. Van Andel et al. (1997) demonstrated that depletion of NK cells (and also the depletion of neutrophils), exacerbated disease with the obligate intracellular bacteria *Clostridium piliforme* in resistant C57Bl/6. The role of neutrophils in protection against intracellular bacteria by lysing infected cells has also been suggested for *L. monocytogenes* (Conlan et al., 1993) and *Francisella tularensis* (Sjostedt et al., 1994). There was evidence of neutrophil trafficking through crypt epithelium in both WT and IFNγR$^{-/-}$ mice with lesions of proliferative enteropathy. Including the presence of neutrophil microabscesses associated with infected proliferative crypts in IFNγR$^{-/-}$ mice (Figure 3.12). At the later time points in the IFNγR$^{-/-}$ mice (when infection with *L. intracellularis* persisted) this infiltration gave way to one of a more chronic nature involving mononuclear cells.

The role of neutrophils in lysing epithelial cells infected with *L. intracellularis* would be worthy of further investigation, particularly since their infiltration into the intestine was noted in both IFNγR$^{-/-}$ and WT mice following inoculation with *L. intracellularis.*

Following immunohistochemical analysis positively stained cells were counted in two ways. Firstly, cell infiltration in the lamina propria was assessed per mm^2 and secondly, in order to count intraepithelial lymphocytes, the number of IEL per 100 epithelial cells was assessed. This investigation of cell infiltration allowed the association of infected and proliferative crypts with the infiltrating cell populations to be explored *in situ.* Analysis of the T cell infiltration in the colon of mice inoculated with *L. intracellularis* in the final challenge study began by looking at the CD3$^+$ population. This ubiquitous T cell marker allows for the initial characterisation of the response involved, and provides a basis on which the following subpopulations can be evaluated. Further assessment of the T cell phenotypes was carried out using antibodies against the surface CD4, CD8 and γδ TCR molecules, as they will help to characterise the response. The expression of the integrin $\alpha_E\beta_7$ (CD103) is associated with intraepithelial lymphocytes, and it was incorporated into the study in conjunction with its epithelial receptor, E-cadherin. Previous observations in experimental animal studies indicated a variation in E-cadherin expression following inoculation with *L. intracellularis* in pigs (MacIntyre, 2001). These changes were not consistent however, and expression was also variable in control animals, therefore further studies in the mouse infection system were deemed prudent.

As mentioned previously animals were not divided into those positive or negative for *L. intracellularis,* but on whether they had been exposed to antigen or not. This approach was taken because it was not possible to monitor the infection over the time course studies, and therefore it was not possible to know whether an animal was resistant to *L. intracellularis* and the disease was never established, or whether they were initially infected and then managed to resolve the infection. It was also not possible to detect whether an animal with detectable *L. intracellularis* displayed a developing or a resolving lesion.

Unbalanced general linear models were used to analyse covariance between inoculation with *L. intracellularis,* and time, for both backgrounds of mice. The null hypothesis was that there would be no significant change in cell infiltration (e.g. $CD3^+$, $CD4^+$ or $CD8^+$ cells) in inoculated, compared with control, mice over the time course studied.

The distribution of $CD3^+$ cells was as expected, with cells found in both the lamina propria and intraepithelial compartments. The pattern of cell infiltration seen in WT mice paralleled the pattern of detectable infection with *L. intracellularis,* with the numbers peaking at day 14, and then returning to normal when the infection was no longer detectable, and presumably the antigenic stimulus had been removed.

Assessment of $CD4^+$ and $CD8^+$ cell infiltration in the final challenge experiment provided the basis for powerful statistical analysis by virtue of the numbers of mice included in the study. Consequently the data could be analysed both by the variation in cell infiltration between inoculated and control animals, and the changing response over time. Immunohistochemical analysis confirmed that the major component of $CD3^+$ infiltrating cells in the lamina propria were also $CD4^+$, and that the IEL component was predominantly $CD8^+$ cells. The data also illustrated that the peak level of cellular infiltration in the lamina propria for inoculated WT mice was at day 14 (for both $CD4^+$ and $CD8^+$ lamina propria lymphocytes).

Comparative analysis of data between WT and $IFN\gamma R^{-/-}$ mice was only viable at day 14 post-inoculation because at days 21 and 28 post-inoculation all WT mice were negative, and all $IFN\gamma R^{-/-}$ mice positive, for *L. intracellularis*. The data would then become an evaluation between those animals positive or negative for disease rather than directly the analysis of animals with different genetic backgrounds. These analyses were obviously of interest, and will help to outline the different and presumably protective response generated in WT mice.

$CD8^+$ and $CD4^+$ T cell responses to inoculation with *L. intracellularis* were initially assessed in WT and $IFN\gamma R^{-/-}$ mice in Experiment three (section 3.2.4) which provided initial data that was later corroborated in a more thorough examination. T cell infiltration was evident in both WT and $IFN\gamma R^{-/-}$ mice, and this concurs with reported data that IFNγ was not required for the generation of a specific immune response (Huang et al., 1993). WT mice did not demonstrate lesions of proliferative enteropathy after day 21 post-inoculation (when the $CD4^+$ count reached its peak), and after this point the number of $CD4^+$ cells also decreased. The $CD4^+$ cell count in infected $IFN\gamma R^{-/-}$ mice was consistently higher than the values obtained for the controls, and remained elevated over time (concomitant with continued *L. intracellularis* infection).

The WT $CD8^+$ T cell response, with the exception of 2 animals at day 21, did not rise significantly above the control values. The $CD8^+$ T cell response in $IFN\gamma R^{-/-}$ mice demonstrated an apparent lag in reaching its maximal value (in relation to $CD4^+$ cell infiltration, this was achieved at day 28) but remained elevated at almost all time points (in comparison to control values). The phenotyping studies carried out demonstrated that the $CD4^+$ and $CD8^+$ cell counts reached their peaks in WT mice 21 days post-inoculation with *L. intracellularis*, and then declined towards the resting control values, in parallel with the absence of PE. The response seen in $IFN\gamma R^{-/-}$ mice challenged with *L. intracellularis*, differed, however, in the sustained presence of $CD8^+$ and $CD4^+$ T cells.

As in the previous experiment this maximal level of $CD4^+$ and $CD8^+$ cell infiltration was associated with the level of infection with *L. intracellularis.* At 21 and 28 days post-inoculation there was no evidence of *L. intracellularis* infection, and at these stages the $CD4^+$ and $CD8^+$ levels were returning to normal resting control values. Both the number and degree of infiltration seen for $CD4^+$ and $CD8^+$ lamina propria lymphocytes were significant for WT mice.

In contrast the changes associated with $CD4^+$ and $CD8^+$ cell infiltration following inoculation in the $IFN\gamma R^{-/-}$ mice was significant only for the changing level of infiltration for $CD4^+$ cells. In this situation the $CD4^+$ lamina propria lymphocytes continued to increase over time as the level of infection with *L. intracellularis* also persisted. For $CD8^+$ lamina propria lymphocyte infiltration there was a trend towards increasing cell number as time progressed, but this increase was slower than that apparent in the WT mice at the earliest time point. $CD8^+$ IELs in the $IFN\gamma R^{-/-}$ mice also appeared to demonstrate a lag period before the level of infiltration began to increase noticeably. *Post-hoc* analysis using the *t*-test illustrated that there was a significant difference in the infiltration of $CD3^+$ IELs and $CD8^+$ cells (in both the lamina propria and the epithelial compartment) between WT and $IFN\gamma R^{-/-}$ mice infected with *L. intracellularis* at 14 days post-inoculation.

This was the only time point at which it was appropriate to compare the level of cell infiltration between infected mice of the two backgrounds, since there was no evidence of infection at any later time point in the inoculated WT group. There were no significant changes at this time point for $CD3^+$ or $CD4^+$ lamina propria cell infiltration between WT and $IFN\gamma R^{-/-}$ mice.

This work did not attempt to determine whether the infiltration of $CD4^+$ T cells seen in response to *L. intracellularis* inoculation was of the Th1 or Th2 type. Monitoring of the cytokines present in the gastrointestinal tract following infection with *L. intracellularis* would be an interesting area of further investigation to elucidate whether a typical Th1 response was generated against the intracellular pathogen or not, and to investigate any differences between the WT and mutant mice.

Another important phenotype of T cells within the colonic mucosa is the $\gamma\delta$ TCR^+ cell population which fulfil a role in both the lamina propria and intraepithelial compartments.

The restricted TCR expression of γδ T cells is an example of differential distribution/phenotype of a lymphocyte subset. This evolutionarily conserved compartmentalisation implies they may also have specialised functions associated with the niche they occupy in the immune system. They contribute to a first line of defence following infection, prior to activation of the conventional adaptive immune response, and bridging the gap between the specific and innate response. There was no obvious change in the γδ TCR^+ cell infiltration in the lamina propria of either $IFN\gamma R^{-/-}$ or WT mice (or between control and inoculated animals). Between 0.5-5% of $CD3^+$ cells express the γδ TCR (Hayday, 2000), and the data presented here is within these parameters. It was also noted that only a few γδ TCR^+ IELs were present and this also corroborates previous data indicating that intraepithelial lymphocytes from the large intestine are predominantly αβ TCR^+, whereas γδ TCR^+ IELs predominate in the small intestine (Beagley et al., 1995). This further demonstrates that as in many areas of intestinal immunology, there are phenotypic variations between cell populations of the small and large intestine, as well as within each region of the gastrointestinal tract. Given the large increase in cellular infiltration following inoculation with *L. intracellularis*, the relative number of γδ TCR^+ cells actually decreases, a feature also seen in a model of spontaneous colitis (Mombaerts et al., 1993b).

γδ TCR^+ cells are the first T cells to develop in an embryonic sense (from day 13 in mice, Carding et al., 1990) and in response to infection in the context of a 'first line of defence' function. Consequently the apparent lack of increased cell infiltration seen following infection with *L. intracellularis* may not suggest that the cells were not involved, merely that their role had already been completed at an earlier stage in infection. In contrast to this however, γδ cells may have a role to play in later immune responses with relation to epithelial repair or immunoregulation. Although this was not indicated for γδ T cells found within the lamina propria, further assessment of the cell responses would ideally also include potential changes in the intraepithelial compartment following inoculation with *L. intracellularis*.

γδ TCR^+ cells, and IELs, as well as 'conventional' αβ TCR^+ lamina propria lymphocytes are involved in the developing immune response by active cytokine secretion in response to various stimuli, such as invading pathogens, which may then direct the ensuing Th1/Th2 response. This balance may be sufficient to result in either the elimination of infection, or an ongoing pathological response. One of the most studied examples of the induction of differing effects of Th responses is that associated with *Leishmania major* infection. In this example, susceptibility is associated with the development of a Th2-dominated immune response, with a Th1-dominated response responsible for resolution of infection (Swihart et al., 1995). In many instances the differentiation of Th precursor cells is influenced by the production of IL-4/IL-12 cytokines, which are involved in influencing the Th2/Th1 response respectively. Data also exists however, for the influence of T cell genetic background on the determination of the default T helper phenotype. Different murine genetic backgrounds can display significantly different polarisation during Th subset development, with this capacity residing with the T cell, and not the APC compartment (Hsieh et al., 1995). For example, susceptibility to *Leishmania* varies among mouse strains as a result of their default Th pathway (and not the absence of precursor cells of either Th subset in the susceptible BALB/c mice, or the resistant C57Bl/6 mice). Similarly these mice do not have a significantly different production level of IL-4 or IL-12, suggesting a role for other genetic factors (Hsieh et al., 1995). Resistance was shown not to be MHC dependent, as different mouse strains, of the same haplotype, exhibited different susceptibilities. The differences in default Th phenotype development due to the background of the T cell resulted in dramatic differences in the activation of a critical macrophage effector function (NO production). Since the host-pathogen relationship is complex and likely to be affected by many genes, the relative importance of default Th phenotype development in any specific *in vivo* situation cannot be absolutely determined. Whether the response to *L. intracellularis* was linked to Th development or MHC haplotype was beyond the scope of this study. The assumption that a protective Th1 response was generated in WT mice is one requiring further investigation.

The establishment of a Th1 or Th2-type helper response, is considered crucial in the development of a protective immune response, in that it defines whether or not the response generated is cell mediated or predominantly humoral. The effect of removing functional IFNγ from the active immune system is an area of much interest and one that presents conflicting data. While some studies have demonstrated that the absence of IFNγ did not result in default to the Th2 pathway (Deckert-Schluter et al., 1996; Swihart et al., 1995), others indicated that there was an increase in IL-4 secreting cells (Szalay et al., 1996; Mountford et al., 1999; Wang et al., 1994). Swihart et al. (1995) illustrated that *Leishmania*-infected IFNγR$^{-/-}$ mice (on a 129/Sv/Ev background) developed a similar Th1 response seen in WT mice, with no expansion of Th2 cells (indicating that IFNγ was not required for the differentiation of CD4^{+} T cell precursors towards a Th1 phenotype, or rather, for down-regulating development of Th2 cells). The resultant exacerbated infection in the mutant mice was attributed to the lack of IFNγ induced production of nitric oxide by the macrophages responsible for the destruction of *Leishmania major.* Wang et al. (1994) however, demonstrated default to the Th2 pathway in the absence of IFNγ (using mice with a targeted disruption of the IFNγ gene on a C57Bl/6 background).

Comparison between the experimental results presented by Swihart et al. (1995) and Wang et al. (1994) were complicated by the different genetic backgrounds of the two knockout mice strains used (IFNγR$^{-/-}$ mice on a 129/Sv/Ev background, and interferon gamma knockouts on a C57Bl/6 background). Previous work by Cantin et al. (1999) suggested that an alternative ligand existed for the IFNγR that was capable of mediating protection against viral challenge. This followed introduction of the IFNγ null mutation into the 129/Sv/Ev mice to enable direct comparisons of infection between IFNγ$^{-/-}$ and IFNγR$^{-/-}$ mice. They further demonstrated that mortality was significantly greater in IFNγR$^{-/-}$ than in IFNγ$^{-/-}$ mice (these two strains are isogenic except for the mutant locus).

Further studies investigated the expression of the integrin $\alpha_E\beta_7$ (CD103) on the infiltrating lamina propria population. There were no significant changes in either the number of $CD103^+$ cells detected following inoculation with *L. intracellularis* or their pattern of infiltration in either WT or $IFN\gamma R^{-/-}$ mice. This compartment was studied to assess the potential for re-directed cytotoxicity of lymphocytes following infection with *L. intracellularis* and the conceivable role for $\alpha_E\beta_7$ as a homing receptor.

There is conflicting experimental data on the precise role of the interaction between $\alpha_E\beta_7$ (CD103) and E-cadherin within the gastrointestinal tract. This is in part due to contradictory findings on the importance of $\alpha_E\beta_7$ (CD103) as either a vital component in homing of the intraepithelial lymphocytes to the gastrointestinal tract, or as a factor in the lymphocytes retention in the mucosa following alternative homing mechanisms. It is still unclear, however, whether this molecule is merely a marker of activated lymphocytes or is actively involved in the IELs homing/retention in the intestinal epithelia (Shibihara et al., 2000).

The functional state of $\alpha_E\beta_7$ on IELs in the normal gut is unclear and it is possible that engagement of E-cadherin on the epithelial cell occurs only after the T cell has been stimulated by antigen (Kilshaw, 1999). Both E-cadherin and $\alpha_E\beta_7$ take part in cell signalling with cross-linking of the integrin co-stimulating the proliferative response and cytotoxic function of IELs (Sarnacki et al., 1991). IELs are abundant in regions of inflamed gut epithelium in which E-cadherin is lacking as a result of genetic manipulation (Hermiston and Gordon, 1995). Therefore suggesting that $\alpha_E\beta_7$ adhesion pathway is not an absolute prerequisite for penetration of lymphocytes into epithelia. It is also known that T cells that infiltrate the gut epithelium in graft versus host disease lack $\alpha_E\beta_7$ (Kilshaw and Baker, 1988) and that the $\alpha_E\beta_7$ integrin appeared to have no significant role in the migration of a primary $CD8^+$ T cell response to viral infection where long-term retention of intestinal intraepithelial lymphocytes was also $\alpha_E\beta_7$ independent (Lefrancois et al., 1999).

Expression of the α_E subunit is also said to be induced by TGFβ (produced mainly by epithelial cells), after T cells have migrated to mucosal tissue (Kilshaw, 1999).

However, these findings are balanced by the fact that $\alpha_E^{-/-}$ mice demonstrate reduced numbers of both intraepithelial and lamina propria T lymphocytes in contrast to normal cell counts seen for Peyer's patch and splenic T lymphocytes. Therefore suggesting that $\alpha_E\beta_7$ was important for generating or maintaining T lymphocytes located diffusely within the epithelium or lamina propria, but not for generating the gut-associated organised lymphoid tissues (Schon et al., 1999).

CD103 expression *in vivo* is usually restricted to previously activated T lymphocytes in the intestinal mucosa, the majority of which belong to the intraepithelial compartment, with a smaller proportion found in the lamina propria. Up-regulation of the $\alpha_E\beta_7$ integrin can occur following stimulation with TGFβ_1, but has not been shown with IFNγ, IL-1α or β, IL-3 or IL-4 (*in vitro,* Parker et al., 1992). The percentage of cells expressing the molecule was also shown to decrease following the withdrawal of TGFβ_1. This indicates that intraepithelial (and perhaps lamina propria) lymphocytes may be capable of responding to epithelial-derived cytokines which result in a change in cell surface adhesion receptor expression and potentially improve both cell function and homing to a specific microenvironment. The absence of any significant up-regulation of $\alpha_E\beta_7$ on the lamina propria lymphocytes (which were assessed to evaluate the potential for re-directed trafficking of lymphocytes towards infected crypts and the intraepithelial compartment) indicates that further work pursuing the expression (and changes in expression) of mucosal TGFβ_1 would be of interest. Investigation of the role of TGFβ1 in the altered proliferation of intestinal enterocytes would also be appealing. Although TGFβ_1 expression is less abundant in the enterocytes of the crypt than those found at the villous tip, alterations in this expression following infection with *L. intracellularis* would be intriguing, particularly considering the evidence of infection with *L. intracellularis* in the epithelial cells found at the tips of the mucosal folds of the colon.

Expression of E-cadherin expression during mouse infection was examined due to its role both as a receptor for $\alpha_E\beta_7$, and in maintaining epithelial cell integrity. Immunohistochemical detection of E-cadherin (Experiment four) demonstrated no notable changes in the expression of the cell adhesion molecule between inoculated or control animals. The level of expression was not consistent among animals, but this has been noted elsewhere (Sloncova et al., 2001; Shun et al., 2001), and is clearly open to interpretation when analysing results. Analysis of altered E-cadherin expression is difficult to quantitate, and as such is guided on a subjective basis. Such methods range from developing internal strong positive controls, followed by the assessment of all sections with relation to this, i.e. cell expression reduced or not reduced (Sloncova et al., 2001). Alternatively results may be graded as normal or abnormal, dependent upon the percentage of positive staining cells within an area of tissue (Shun et al., 2001). In this study all sections of colon were assessed on the fundamental expression of E-cadherin, and were all found to be positive.

The reduction in expression of E-cadherin seen in epithelial cells following infection with *H. pylori* is associated with disease progression and the development of gastric carcinoma (Terres et al., 1998). Down-regulation of E-cadherin coincides with the transition from well-differentiated adenoma to invasive carcinoma (Perl et al., 1998) and the expression of E-cadherin is reduced in 45.5% of cancers of various organs (Shiozaki et al., 1991). This correlation between E-cadherin down-regulation and metastasis might be related to both the ability of E-cadherin-negative cells to invade surrounding tissue and also the increased likelihood of weakly adherent cells to detach from the tumour mass in response to low shear forces (such as those found in lymphatic vessels and venules; Byers, 1995). The staining intensity seen in cancer tissues was lower than normal tissues at the cell boundaries, but showed evidence of increased E-cadherin expression throughout the cytoplasm (Inoue et al., 1992), indicating that expression patterns may be affected by malignant transformation of the cell.

L. intracellularis infection, while linked with increased epithelial proliferation, has not been associated with carcinomatous lesions, and so the absence of significant changes in the expression of E-cadherin is not entirely unexpected.

B lymphocytes.

Initial work in Experiment four attempted to analyse B cells involved in the mucosal immune response by immunohistochemical detection. As presented in the Results there was successful detection of $B220^{+}$ cells in frozen colonic sections, but this was found to be mainly associated with lymphoid follicles, with very few cells situated throughout the lamina propria. Consequently the MGP stain was carried out to provide a more expansive study and to distinguish plasma cells within this region of the gastrointestinal tract. Accordingly this data illustrated that there was a significant increase in the number of plasma cells associated with areas of crypt cell proliferation in the $IFN\gamma R^{-/-}$ mice inoculated with *L. intracellularis,* and this became increasingly prominent as time progressed. They were apparent as focal aggregates of plasma cells within surface lamina propria at the basolateral surface of the epithelial cells and surrounding proliferative crypts in the deeper lamina propria. Significant plasma cell infiltration was not a feature associated with WT mice inoculated with *L. intracellularis*, although there was a slight increase in cell numbers in comparison to the control mice.

Although IgA constitutes only a small fraction of serum immunoglobulins, secretory IgA is the predominant immunoglobulin found in external secretions such as the mucus of the gastrointestinal tract. The plasma cells that secret IgA home to subepithelial tissue. The IgA then binds to the epithelial expressed poly-Ig receptor where the complex is transported across the epithelial barrier into the lumen. Following enzymatic cleavage of the poly-Ig receptor from the receptor, it then forms the IgA secretory component and is released, together with polymeric IgA, into the mucous secretions. It was assumed that the accumulation of plasma cells seen in mice following inoculation with *L. intracellularis* were IgA^{+} cells, although confirmation of this is required.

In support of this accumulation of IgA has been noted in intestinal enterocytes of pigs following experimental challenge with *L. intracellularis* (McOrist et al., 1992). Also of interest is that in a study performed by Kamata et al., (2000) an increased frequency of surface IgA-positive plasma cells in the intestinal lamina propria in a murine model of IgA nephropathy was demonstrated. In particular they illustrated that the majority of $B220^+$ cells were IgM^+, and only a small number of IgA^+B220^+ cells were found in the lamina propria. This finding might further imply that the plasma cells detected here were IgA^+ cells, considering the associated lack of $B220^+$ cell expression. Kamata et al., (2000) also demonstrated that the frequency of IgA-plasma cells in the intestinal lamina propria was a critical determinant of the serum IgA level in rodents. In fact a major source of serum IgA expression in rodents has long been suggested to originate in large part from polymeric IgA produced in the intestine (Vaerman et al., 1973). The level of serum IgA is dependent not only on production and catabolism but also on the excretion into the gut lumen via intestinal epithelium and biliary tract (Hendrickson et al., 1995). Consequently confirmation of the plasma cell type in the intestinal tract following inoculation with *L. intracellularis*, and the associated response in the serum of these animals would be worthy of further investigation.

Preliminary work demonstrating a serological response carried out by Rowland and Lawson (1974) illustrated that serum from an animal with proliferative enteropathy recognised bacteria in the apical cytoplasm of the immature enterocytes of the ileum and colon. Later work demonstrated a specific antibody response in hamsters suffering from proliferative enteropathy (Jacoby, 1978), which also recognised intracellular organisms. In the hamster study all animals with lesions of PE had a demonstrable serological response from day 10 after infection. Further studies by Lawson and McOrist (1993) demonstrated that IgM was detected for only a brief period (4-6 weeks) in association with intestinal lesions of PE in the pig. Previously they had illustrated *L. intracellularis* specific IgA and IgM responses within the serum of infected pigs (Lawson et al, 1988), but a weak IgG response. Evidence therefore exists for the establishment of a humoral response following infection with *L. intracellularis.*

Further characterisation of the antibody response was required however, to determine the specific antigens of *L. intracellularis* recognised. As detailed previously (3.4) circulating antibodies from mice recognised a variety of proteins following separation of *L. intracellularis* on a polyacrylamide gel, and western blot analysis. The response generated by both wildtype and IFNγR$^{-/-}$ mice (Experiment three) was analysed following inoculation with *L. intracellularis.* Blood samples were collected from all animals (both those challenged with *L. intracellularis* and control), immediately prior to necropsy (2.2.1.4). The results demonstrated unequivocally that there was no recognition of *L. intracellularis* antigens in any control animals inoculated with SPG buffer (Figures 3.44). In contrast the response seen in all animals inoculated with *L. intracellularis* showed signs of seroconversion against the 25-27 kDa immunodominant proteins. Recognition of the organism was seen in all animals inoculated with *L. intracellularis*, both WT and IFNγR$^{-/-}$, and was not restricted to those with lesions of proliferative enteropathy. The response was seen at the first time point examined (14 days post-inoculation), and remained present until the final time point of 35 days. A variety of antigens were recognised by plasma of inoculated animals (Figure 3.44) but recognition of the 25-27kDa proteins was the most consistent feature.

The persistent reactivity of the plasma samples from animals inoculated with *L. intracellularis*, irrespective of the presence of demonstrable lesions of PE at necropsy, indicated that the transient nature of infection seen in WT mice (compared to the prolonged disease seen in IFNγR$^{-/-}$ mice), was not represented by a declining serological response following resolution of infection.

The secondary antibodies used for western blot analysis in this study recognised all mouse IgG subclasses, IgA, and IgM. Further clarification of the immunoglobulin isotypes recognised would be of interest, particularly regarding the antibody response generated in IFNγR$^{-/-}$ mice.

Previous studies demonstrated that while IFNγR$^{-/-}$ mice were capable of generating a serological response to antigenic stimuli (Huang et al., 1993), mice lacking a functional IFNγ system had decreased total serum IgG2a (associated with a Th1 response), but generated a level of IgG1 comparable to WT. There was evidently a modulatory role played by IFNγ in the production of IgG isotypes. Huang et al. (1993) further demonstrated that a similar isotype pattern was observed in IFNγR$^{-/-}$ mice in comparison to WT, indicating that IFNγ was involved in immunoglobulin isotype regulation and enhancement of the generation of a normal IgG2a response, even though it seems nonessential for switching to this isotype. Interestingly the B cell response to *T. gondii* was normal in IFNγR$^{-/-}$ mice with apparently unimpaired antibody production (IgM and IgG), Deckert-Schluter et al., 1996, in both WT and IFNγR$^{-/-}$ mice.
Conflicting data, however, suggests that while functional Th cells are required for IgG2a production in response to infection, this is not dependent on a functional IFNγ signalling pathway (MarkineGoriaynoff et al., 2000).

While, classically, cellular and not humoral immunity is considered important in protection of the host from infection with intracellular bacteria, some studies have indicated a role for antibody. Winslow et al. (2000) demonstrated that while cellular immunity was required for elimination of infection with the obligate intracellular bacteria *Ehrlichia chaffeensis*, antibodies played a role in aiding bacterial clearance (although *E. chaffeensis* is more exposed to the serum immune response following infection of circulating leucocytes).

Antibodies have an established function in neutralising extracellular bacterial infection, and in preventing attachment. These functions, however, are not applicable to the lifecycle of an intracellular pathogen. Exposure to the external milieu would be required, and this is not a recognised feature of the pathogenesis of *L. intracellularis*.
The role of antibody in neutralising viruses within cells has been postulated (Levine et al., 1991), and so a similar role against intracellular bacteria may also be possible.

As mentioned previously, McOrist et al. (1992) have documented the accumulation of IgA within enterocytes infected with *L. intracellularis* in porcine PE. The specificity of the antibody was not determined, however, and so may have been as a result of inefficient transcytosis of the immunoglobulin by the enterocyte due to infection with the intracellular organism, rather than as a specific response directed against the pathogen. The role of antibody in protection of the host from infection with *L. intracellularis* was not determined in this study, although the analysis has provided corroboratory evidence for a pathogen-specific response to *L. intracellularis.* Further studies, perhaps involving passive transfer of antibodies to immunosuppressed mice, may help to distinguish a function for the specific antibodies generated in response to infection. Further studies into the developing serological response were initiated in Experiment four. Plasma samples were collected from all mice prior to inoculation, and then at weekly intervals until the end of the study. The projected aim was to assess the developing serological response as both time and the disease process developed. Unfortunately this study was not completed, but preliminary results suggested that there was a detectable IgG response in inoculated IFNγR$^{-/-}$ mice at the first two time points (days 14 and 21 post-inoculation), but the isotype was not determined.

Future work to aid in characterisation of the antibody response would include not only elucidation of the serological response and the isotypes generated following infection with *L. intracellularis*, but also the titres of antibody generated and possible immunogens of *L. intracellularis*. Assessing the kinetics of IgG, IgA, and IgM production in the sera, and mucosa, of infected animals would lead to further clarification of the generated immune response to infection with *L. intracellularis,* and the affect that IFNγ may have on these pathways.

4.6 Summary and Conclusions.

In summary, the work presented in this thesis has established a mouse model for proliferative enteropathy in 129/Sv/Ev and isogenic IFNγR$^{-/-}$ mice following oral challenge with *Lawsonia intracellularis*. Although this is an important pathogen in pigs there is little detailed information regarding the immune response generated in the host and this study is the first to examine, in detail, the response to infection with this unique obligate intracellular bacterium. Due to this intracellular lifestyle Th1-type responses may be important in the elimination of infection, and so IFNγR$^{-/-}$ mice were incorporated into the analysis to evaluate the role of this vital Th1 cytokine. The present study demonstrates that deficiency of the IFNγR did render mice highly susceptible to *L. intracellularis* infection, with a more pronounced infection apparent than that seen in WT mice. The disease that developed in both backgrounds of mice was characteristic of porcine PE, with hyperplasia of immature crypt epithelial cells, *L. intracellularis* found multiplying freely within the apical cytoplasm of the enterocytes, and an associated loss of goblet cells. In both WT and IFNγR$^{-/-}$ mice with lesions of PE, the organism demonstrated a tropism for colonic enterocytes. Increases in the number of enterocytes per crypt, and the decrease of associated goblet cells were statistically significant. Notably the number of enterocytes per crypt was significantly higher in inoculated IFNγR$^{-/-}$ than WT mice. This demonstrates that the absence of a functional IFNγ signalling pathway affects not only the hosts' response to infection with *L. intracellularis*, but that regulation of epithelial cell growth and differentiation is also affected. This is indicative of a role for the immune response in both defence and maintenance of epithelial homeostasis.

Analysis of the immune response following inoculation with *L. intracellularis* revealed that a notable inflammatory response, initially of neutrophils, and later replaced with mononuclear cells (indicating chronic inflammation), was elicited in both WT and IFNγR$^{-/-}$ mice challenged with *L. intracellularis* in all challenge studies, a feature not previously noted.

The initial phenotyping study (3.2.4) suggested the T cell response in the lamina propria consisted predominantly of $CD4^+$, rather than $CD8^+$ cells. The study also indicated that in WT mice the cell infiltration returned to normal control values concomitantly with the absence of detectable *L. intracellularis*. In inoculated $IFN\gamma R^{-/-}$ mice there appeared to be a less marked infiltration of $CD4^+$ cells in comparison to the WT mice, but the pattern of cell kinetics was similar. This was in contrast to the $CD8^+$ cell response that appeared comparatively delayed.

The spectrum of expressed antigens examined was expanded in the final challenge study permitting both more detailed analysis of T cell subsets and more in depth study of associated immunological parameters. Significantly, the numbers of mice used in this study provided statistically powerful data. As detailed previously there was prolonged cellular infiltration in the $IFN\gamma R^{-/-}$ mice associated with the continuing infection with *L. intracellularis*. The most notable differences between the two backgrounds of mice focus on day 14 post-inoculation, the only time point at which *L. intracellularis* was detected in both groups. These analyses demonstrated a significant reduction in the number of infiltrating $CD8^+$ lymphocytes in both the lamina propria and intraepithelial compartments of $IFN\gamma R^{-/-}$ mice. A significant difference was also noted in the $CD3^+$ intraepithelial sector, but not in the lamina propria (where the lymphocytes were predominantly $CD4^+$). Although the basis of these antigen-dependent interactions requires characterisation, the implication is clearly that a specific immune response was generated in both WT and $IFN\gamma R^{-/-}$ mice following infection with *L. intracellularis*, which is in contrast to previous reports of experimental proliferative enteropathy. Furthermore the extent of this response is significantly different in $IFN\gamma R^{-/-}$ mice demonstrating that absence of the IFNγR adversely affects the hosts ability to eliminate disease.

Although the activation status of the infiltrating cell population was not ascertained, it would be interesting to investigate this aspect since, for example, the absence of a functioning IFNγ signalling pathway is known to have an adverse effect on macrophage function (Deckert-Schluter et al., 1996). The reduction in infiltrating $CD8^+$ lymphocytes in $IFN\gamma R^{-/-}$ mice at the earliest time point (day 14), is a factor not noted in other experimental systems (Deckert-Schluter et al., 1996; Huang et al., 1993). As such, although *L. intracellularis* may be predicted to ultimately induce the typical Th1 response normally associated with intracellular bacterial infections, the time course of this and the 'downstream' events may be specifically altered in view of modifications in the inherent functional capacity of the induced effector cells. The possible Th1 dependent immune response following infection with *L. intracellularis*, backed by the infiltration of macrophages and $CD8^+$ lymphocytes to sites of infection, as well as the disruption in $IFN\gamma R^{-/-}$ mice, indicates a possible role for uninhibited Th2 lymphocytes in resultant pathology. Further studies to assess the development of a Th1 or Th2 type response following *L. intracellularis* inoculation in WT mice might utilise antibodies that detect the IFNγR2. This receptor is most commonly associated with Th2 cells, and enables IFNγ to inhibit Th2 proliferation, and thereby promote a Th1 type response (Pernis et al., 1995).

As a result of these studies it would be pertinent for future challenge studies to include earlier assessment of the cell infiltration following inoculation, even if the levels of *L. intracellularis* were undetectable. This would allow both a more complete chronological study of the pathological changes induced by *L. intracellularis* and analysis of the infiltration patterns of immune cells that may be involved in the earlier responses to infection. For example, γδ TCR^+ cells and intraepithelial lymphocytes, as mentioned previously, do not respond to stimulation transmitted via the classic TCR/CD3 pathway, and may bridge the gap between innate and specific immunity by recognising conserved antigens. Consequently assessment of early responses following inoculation will supplement this emerging picture.

Specifically this might incorporate additional analyses at days 3, 7, 10, and 17 post-inoculation. Based on the findings presented here it would be appropriate to evaluate IL-2R expression to confirm adequate activation of T cells (seen by Deckert-Schluter et al., 1996), and also to incorporate *in vitro* assays to demonstrate the specificity of the lymphocyte response for *L. intracellularis* (corroboratory evidence for which has already been found in the specific serological response). Evaluation of the mucosal response following infection should also incorporate analysis of the cytokine profiles in the intestine (to further elucidate the Th1/Th2 response) and also the expression of growth factors such as KGF and their involvement in the regulation of epithelial cell proliferation. In particular TNFα is known to increase myofibroblast production of KGF, but its synthesis is impaired in IFNγR$^{-/-}$ mice (Kamijo et al., 1993a), which also has implications for the activation of microbicidal activity and nitric oxide release in macrophages.

The presumption that all animals inoculated with *L. intracellularis* responded to the pathogenic stimulus, even if the organism was not detected (or had already been eliminated) was corroborated by the cell infiltration patterns and the alterations in goblet cell numbers in WT mice. For example at day 14 the values demonstrate marked changes from those of the control and by day 28 they had mainly returned to normal. The most telling values here in this regard, are those at day 21 where the cells appear to be midway between the two values, indicating that they were involved in an earlier response, but removal of the stimulus results in the gradual return to equilibrium.

Analysis of the serological response in the mouse following inoculation with *L. intracellularis* also demonstrates that a specific response was seen in all animals inoculated with *L. intracellularis* (irrespective of either the presence *L. intracellularis*, or a functional IFNγR). They further illustrated that the antibodies generated were principally directed against the apparent 25-27 kDa immunodominant antigens.

In addition to this systemic response a role for mucosal plasma cells was also evident in the later stages of infection in IFNγR$^{-/-}$ mice infected with *L. intracellularis* that was not apparent in WT mice. The recruitment of these cells to longer term lesions, and their continued proliferation is consistent with continuing infection and failure of successful elimination by T cells and macrophages.

This work has provided the most detailed analysis and dissection of the immune response (and associated pathological feature) as a consequence of *L. intracellularis* infection that has been accomplished to date in any species. In addition to the relevance of these findings as regards the specific immune response associated with this bacterium, it also provides us with a unique model system to study both mechanisms of immunity to intracellular bacterial pathogens and the associated epithelial cell dysregulation.

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APPENDIX I

Agar Scientific Limited	Essex, UK.
Amersham International	Slough, UK.
BDH Merck	Poole, Dorset, UK.
BD PharMingen	Heidelberg, Germany.
Biorad	Hemel Hempstead, Herts., UK.
Bibby Sterilin	Staffordshire, UK.
Boehringer Mannheim	Lewes, Sussex, UK.
British Biocell International	Cardiff, UK.
Dako	Glostrup, Denmark.
GibcoBRL	Paisley, UK.
ICN Biomedicals Ltd.	Hampshire, UK.
Leo Laboratories Ltd.	Buckinghamshire, UK.
Santa Cruz Biotechnology Inc.	Calne, UK.
Serotec	Oxford, UK.
Shandon	Pittsburgh, USA.
Sigma	Poole, Dorset, UK.
Surgipath	Peterborough, UK.
Tissue Tek	Miles, USA.
Vector Laboratories	Peterborough, UK.

Appendix II.

Histopathology – IFNγR$^{-/-}$ mice, day 14 post-inoculation.

Mouse I/D		ILEUM				COLON		
	%infection	Lamina propria inflammation	Epithelium	Other comments	% infection	Lamina propria	Epithelium	Other comments
A1-1	24.6	+ L, PMN, PC, M	Focal crypt hyperplasia/ GC loss		61.9	+ L, PMN, PC, M	Marked diffuse crypt hyperplasia. Loss of GC. PMN traffic into crypt lumens	Submucosal PMN infiltrate
A1-2	22.1	+ L, PMN, PC, M	Focal crypt hyperplasia/ GC loss		86	+ L, PMN, PC, M	Marked diffuse crypt hyperplasia. Loss of GC. PMN traffic into crypt lumens	Submucosal L, PMN, M
A1-3	20.9	+ → +++ L, PMN, PC, M	Focal crypt hyperplasia/ GC loss		35	+ / ++ L, PMN, PC, M	Marked diffuse crypt hyperplasia. Loss of GC. Occasional PMN traffic into crypt lumens	
A1-4	25	-/+ L, PMN, PC, M	Focal crypt hyperplasia/ GC loss		48.7	+ L, PMN, PC, M	Marked diffuse crypt hyperplasia. Loss of GC.	
A1-5	2.5		One hyperplastic crypt/ GC loss		46.1	+ L, PMN, PC, M. one focus L++	Multifocal crypt hyperplasia/ GC loss. PMN traffic into crypt lumens	

A1-6	48.1	-/+ L, PMN, PC, M	Focal to confluent crypt hyperplasia/ GC loss					NAD
A1-7	0.7				4	-/+ L, PMN, PC, M	Multifocal crypt hyperplasia and GC loss	
A1-8	2		One hyperplastic crypt/ GC loss		0.7			NAD
A1-9	46.9	+ L, PMN, PC, M	Extensive crypt hyperplasia and GC loss		14.9	-/+ L, PMN, PC, M	One extensive focus crypt hyperplasia/ GC loss	
A1-10	1.2		One hyperplastic crypt/ GC loss		0			NAD

L: lymphocytes
PMN: polymorphonuclear phagocytes
PC: plasma cells
M: macrophages
GC: goblet cells

NAD: no abnormalities detected

Note :
1) normal control gut contains L, M, PC and PMN as part of 'resident' inflammatory component.
-/− = normal to very mild increase beyond normal
\+ = mild increase beyond normal
+− = moderate increase beyond normal
+−+ = marked increase beyond normal
2) Results relate to general semi-quantitative histopathological description of lesions

Histopathology – IFNγR$^{-/-}$ mice, day 21post-inoculation.

Mouse I/D (Timepoint)		ILEUM				COLON		
	%infection	Lamina propria	Epithelium	Other comments	% infection	Lamina propria	Epithelium	Other comments
A2-1	1.8		One hyperplastic crypt/ GC loss		21.6	+ L, PMN, PC, M Focal L++	Large foci crypt hyperplasia/ GC loss	
A2-2	33.6	+/++ L, PMN, PC, M	Many hyperplastic crypts/GC loss		84	+/+++ L, PMN, PC, M	Large foci crypt hyperplasia/ GC loss	Submucosal L, M, PMN ++
A2-3					32.8	++/+++ L, PMN, PC, M	Large foci crypt hyperplasia/ GC loss. Small crypt abscesses	
A2-4	54.6	+/++ L, PMN, PC, M	Stunting/ fusion of villi; crypt hyperplasia/ GC loss		14.9	+/++ L, PMN, PC, M	Large foci crypt hyperplasia/ GC loss	Submucosal L, M, PMN ++. Extends through muscularis to serosa and adherent mesenteric fat.
A2-5	92	+/++ L, PMN, PC, M	Stunting/ fusion of villi; extensive crypt hyperplasia/ GC loss		48.1	++ L, PMN, PC, M	Large foci crypt hyperplasia/ GC loss	
A2-6	53	+/++ L, PMN, PC, M	Stunting/ fusion of villi; extensive crypt hyperplasia/ GC loss		51	+++ L, PMN, PC, M	Large foci crypt hyperplasia/ GC loss. Small crypt abscesses	
A2-7	6.5		Focal crypt hyperplasia/ GC loss		11.6	-/+ L, PMN, PC, M	Occasional foci crypt hyperplasia/ GC loss.	
A2-8	24	-/+ L, PMN, PC, M	Focal crypt hyperplasia/ GC		61.3	+++ L, PMN, PC, M	Extensive crypt hyperplasia/ GC	Focal submucosal L,

			loss				loss.	M ++
A2-9	26.3		Focal crypt hyperplasia/ GC loss		72	+/++ L, PMN, PC, M	Extensive crypt hyperplasia/ GC loss. Occasional intraepithelial PMN aggregates	
A2-10	21.2	+ L, PMN, PC, M	Focal crypt hyperplasia/ GC loss. Focal microabscesses		74.2	++/+++ L, PMN, PC, M	Extensive crypt hyperplasia/ GC loss. Occasional intraepithelial PMN aggregates	Submucosal L, M, PMN ++

Histopathology – IFNγR$^{-/-}$ mice, day 28 post-inoculation.

Mouse I/D		ILEUM				COLON		
	%infection	Lamina propria inflammation	Epithelium	Other comments	% infection	Lamina propria	Epithelium	Other comments
A3-1	4.6	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss		81.1	++/+++ L, PC, M PC ++ at surface	Surface flattening. Extensive hyperplasia/ GC loss	
A3-2	11.9	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss		92.3	++ L, M PC +++ diffuse	Surface flattening. Extensive hyperplasia/ GC loss	Submucosal M, PC+++
A3-3	0.45	PMN ++ (focal)	Focal hyperplasia/ GC loss		22.9	+++ L, PMN, PC, M PC++ surface	Surface flattening. Extensive hyperplasia/ GC loss	Some depletion of epithelial (crypt elements by the extreme inflammation)
A3-4	5.2	Active GALT	Focal hyperplasia/ GC loss		87.8	+++ L, PMN, PC, M	Surface flattening. Extensive hyperplasia/ GC loss	
A3-5	27.5	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss		95	+-/+++ L, PMN, PC, M Surface plasma cells ++	Surface flattening. Extensive hyperplasia/ GC loss. Debris in crypts	Submucosal L, M, PC

A3-6	2.1			Extensive autolysis				Extensive autolysis
A3-7	76.4	+++ L, PMN, PC, M	Extensive hyperplasia/ GC loss. Flattening of surface		20.3	+/++ L, PMN, PC, M	Focal hyperplasia/ GC loss	
A3-8	51.4	++/+++ L, PMN, PC, M L ++ focal	Crypt hyperplasia/ GC loss. Crypt distension					
A3-9	62.4	+/++ L, PMN, PC, M	Extensive crypt hyperplasia/ GC loss.		75.9	+/++ L, PMN, PC, M	Focal hyperplasia/ GC loss	
A3-10					87.7	Depletion, fibrosis, occasional foci PMNs. M+	Extensive hyperplasia/GC loss	Submucosal oedema, PMN ++, plasma cells ++

Histopathology – WT mice, day 14 post-inoculation.

Mouse I/D		ILEUM				COLON		
	%infection	Lamina propria inflammation	Epithelium	Other comments	% infection	Lamina propria	Epithelium	Other comments
B1-1				NAD				NAD
B1-2	7.8	-/+ L, PMN, PC, M	Occasional hyperplastic crypt/ GC loss. Focal degenerate intraepithelial cells		6.2	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss	
B1-3	3.5	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss			-/+ L, PMN, PC, M		
B1-4	2.4	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss.			-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss. Debris in crypt lumen	
B1-5	19.7	+ L, PMN, PC, M	Scattered hyperplasia/ GC loss		4.36	+/++ L, PMN, PC, M	Focal hyperplasia/ GC loss. Debris in crypt lumen	
B1-6	6.4	++ L, PMN, PC, M	Scattered hyperplasia/ GC loss		2	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss	
B1-7	19.2	++ L, PMN, PC, M	Scattered hyperplasia/ GC loss					NAD

B1-8	6.99	+ L, PMN, PC, M	Focal hyperplasia/ GC loss		8.2	+ L, PMN, PC, M	Focal hyperplasia/ GC loss	
B1-9	1.65		Focus hyperplasia/ GC loss		18.5	-/+ L, PMN, PC, M	Scattered hyperplasia/ GC loss	
B1-10	7	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss		4.5	-/+ L, PMN, PC, M	Focal hyperplasia/ GC loss	

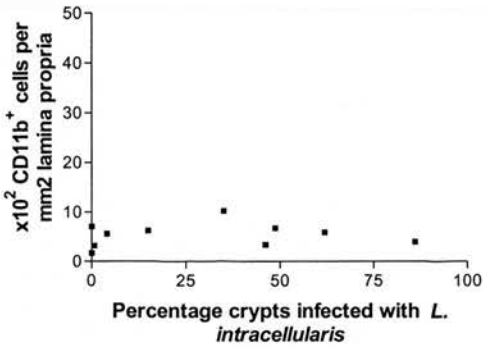
Histopathology - WT mice, day 21 post-inoculation.

Mouse I/D	ILEUM			COLON		
	Lamina propria inflammation	Epithelium	Other comments	Lamina propria	Epithelium	Other comments
B2-1			Mod. autolysis			NAD
B2-2			NAD	L+ (focal)		
B2-3	+/++ L, PMN, PC, M			-/+ L, PC, M		
B2-4	+/++ L, PC, M					
B2-5	-/ + L, PC, M			+ L, PMN, PC, M		
B2-6	GALT hyperplastic. Abscess in GALT			-/ + L, PC, M		Scattered submucosal mixed +
B2-7	+ L, PMN, PC, M			+/++ L, PMN, PC, M		
B2-8			Mod. autolysis			Focal GC hyperplasia
B2-9	+ L, PMN, PC, M			+ L, PMN, PC, M		
B2-10				Focal L+		

Histopathology - WT mice, day 28 post-inoculation.

Mouse I/D	ILEUM			COLON		
	Lamina propria inflammation	Epithelium	Other comments	Lamina propria inflammation	Epithelium	Other comments
B3-1			NAD			NAD
B3-2			NAD			NAD
B3-3			NAD	Focal L+		
B3-4	-/+ L, PMN, PC, M					NAD
B3-5			NAD			NAD
B3-6			NAD			NAD
B3-7	Focal L +					NAD
B3-8			NAD			NAD
B3-9			NAD			NAD
B3-10			NAD			NAD

APPENDIX III



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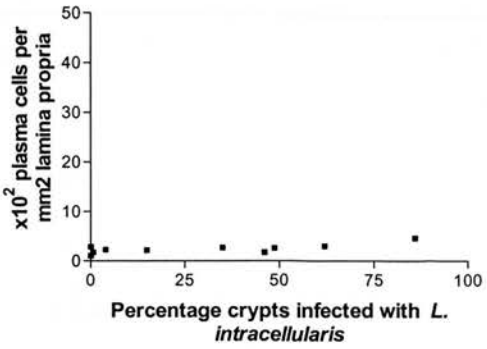

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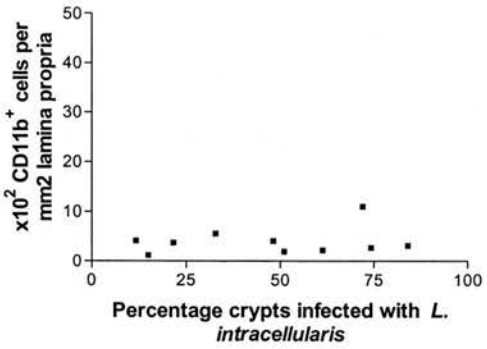

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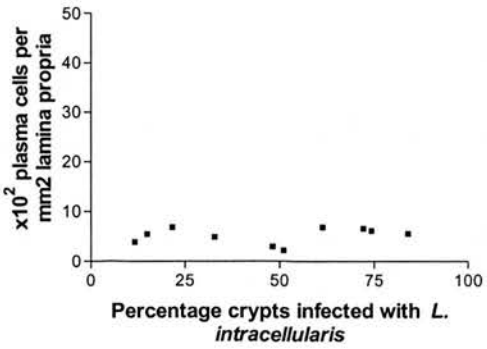

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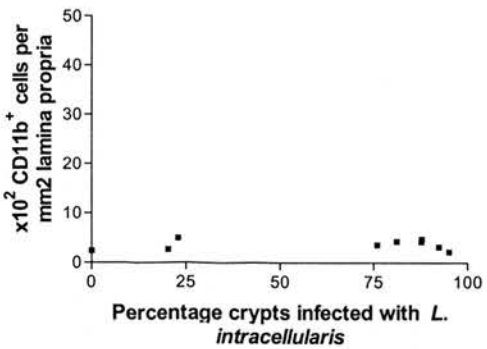

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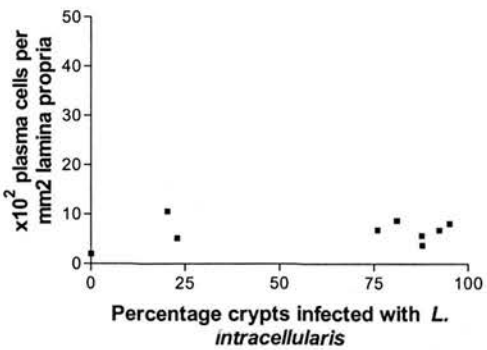
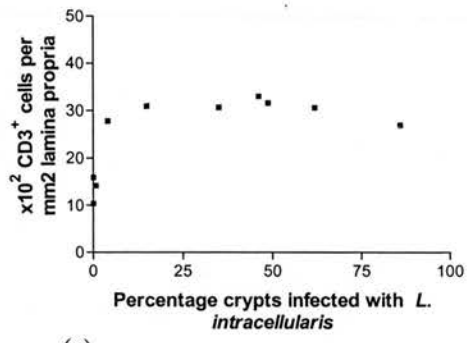


(f)

Appendix III. Correlation graphs for $CD11b^+$ and plasma cell infiltration in inoculated $IFN\gamma R^{-/-}$ mice. Figures (a), (c) and (e) represent the $CD11b^+$ lamina propria infiltration at days 14, 21 and 28 post-inoculation respectively.
Figures (b), (d) and (f) represent the plasma cell lamina propria infiltration at days 14, 21 and 28 post-inoculation respectively.

APPENDIX III



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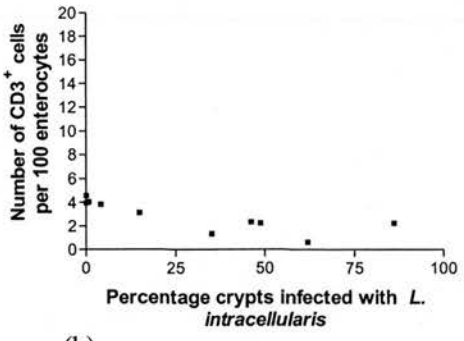

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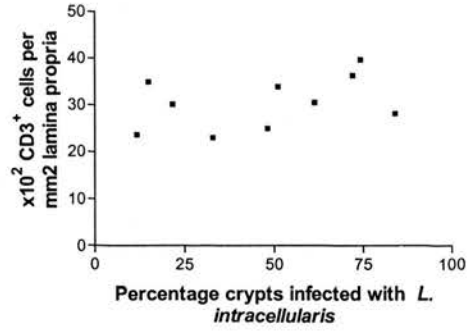

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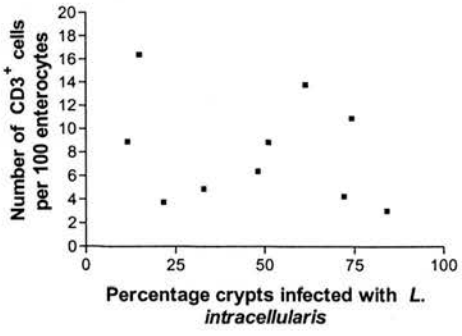

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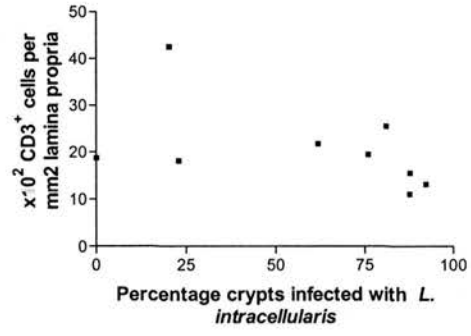

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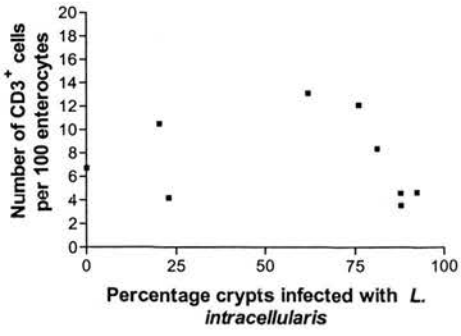
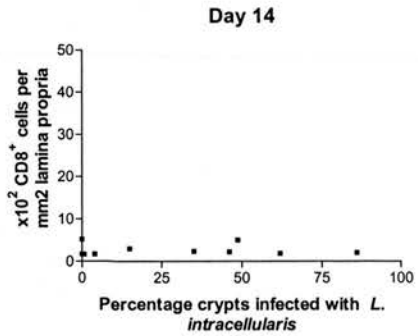


(f)

Appendix III. Correlation graphs for $CD3^+$ cell infiltration in $IFN\gamma R^{-/-}$ mice inoculated with *L. intracellularis* at days 14 (a, b), 21 (c, d) and 28 (e, f) post-inoculation.
(a), (c) and (e) – $CD3^+$ cells per mm2 lamina propria,
(b), (d) and (f) – $CD3^+$ cells per 100 enterocytes.

APPENDIX III



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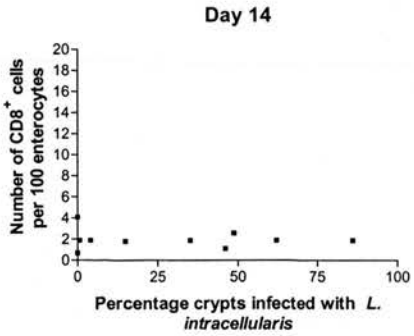

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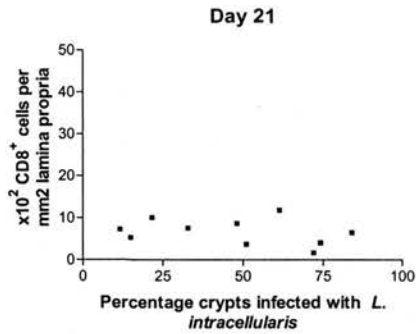

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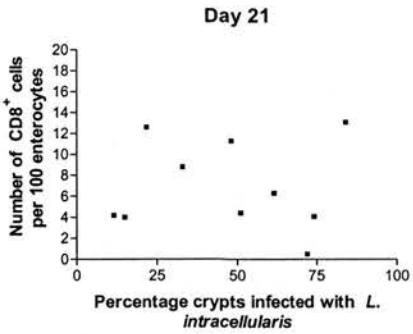

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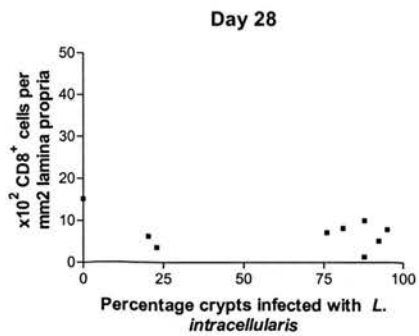

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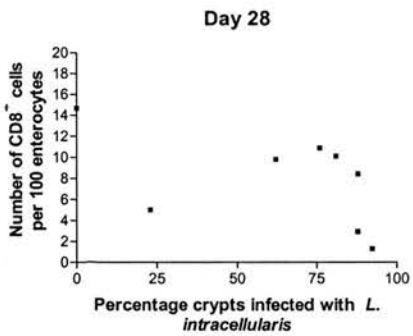
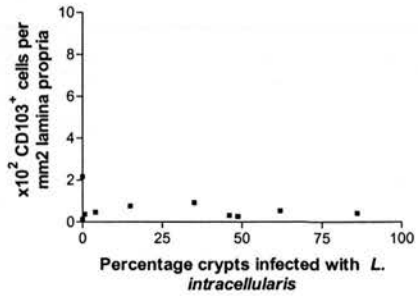


(f)

Appendix III. Correlation graphs for $CD8^+$ infiltration in inoculated $IFN\gamma R^{-/-}$ mice.
Figures (a), (c) and (e) represent the $CD8^+$ lamina propria infiltration at days 14, 21 and 28 post-inoculation respectively.
Figures (b), (d) and (f) represent the number of $CD8^+$ cells per 100 enterocytes for days 14, 21 and 28 post-inoculation respectively.

APPENDIX III



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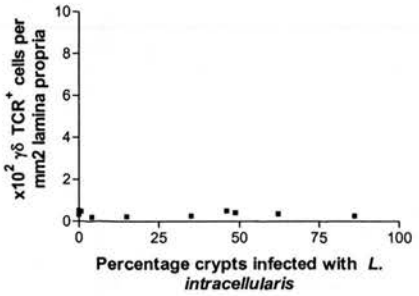

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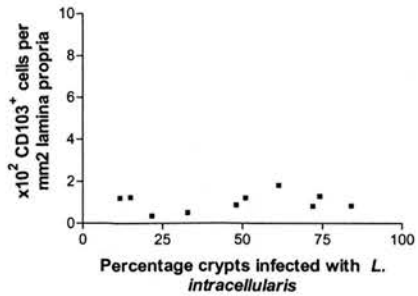

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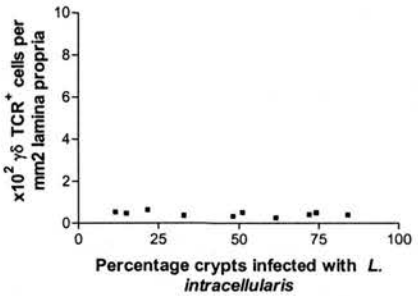

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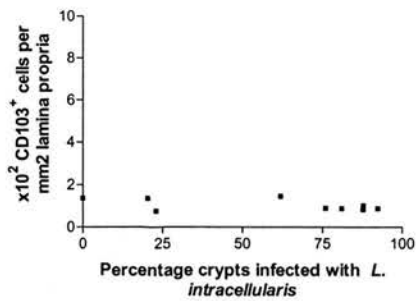

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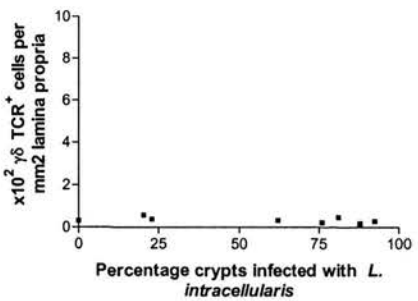
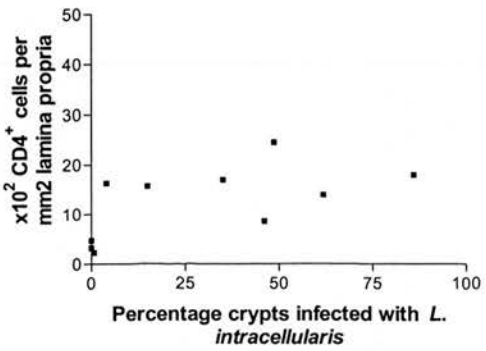


(f)

Appendix III. Correlation graphs for $CD103^+$ and $\gamma\delta$ TCR^+ T cell lamina propria infiltration in $IFN\gamma R^{-/-}$ mice following inoculation with *L. intracellularis*.
Figures (a), (c) and (e) represent the $CD103^+$ infiltration at days 14, 21 and 28 post-inoculation respectively.
Figures (b), (d) and (f) represent the $\gamma\delta^+$ infiltration at days 14, 21 and 28 post-inoculation respectively.

APPENDIX III



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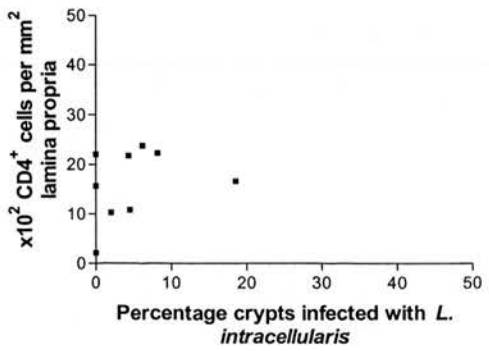

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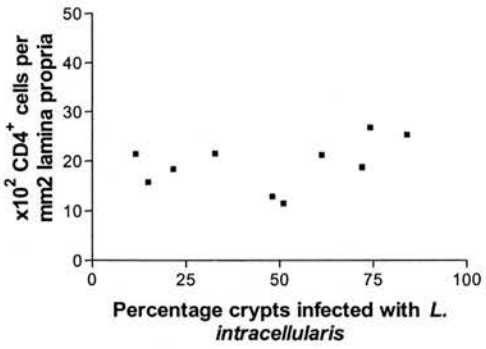

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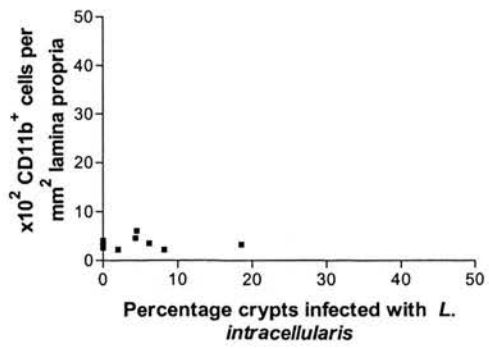

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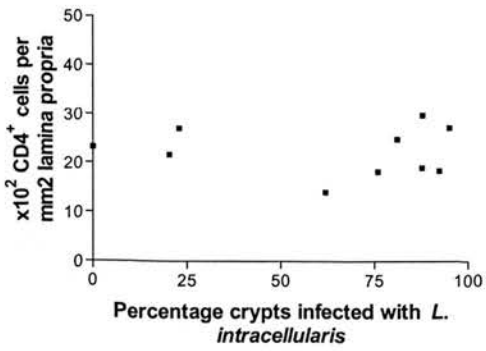

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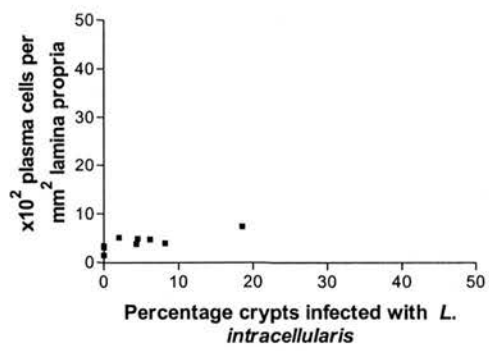
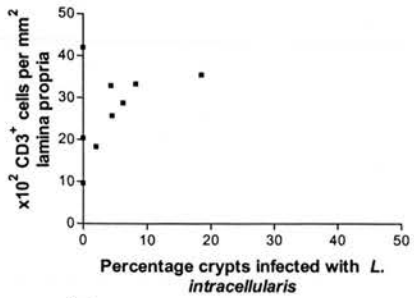


(f)

Appendix III. Correlation graphs for both WT and IFNγR$^{-/-}$ mice inoculated with *L. intracellularis*.
Figures (a), (c) and (e) represent the CD4$^+$ lamina propria infiltration in inoculated IFNγR$^{-/-}$ mice at days 14, 21 and 28 post-inoculation respectively.
Figures (b), (d) and (f) represent the lamina propria infiltration in inoculated WT mice at day 14 for CD4$^+$, CD11b$^+$ and plasma cells respectively.
Note the different scales for the x-axis of the WT and IFNγR$^{-/-}$ graphs due to the lower proportion of crypts infected with *L. intracellularis*.

APPENDIX III



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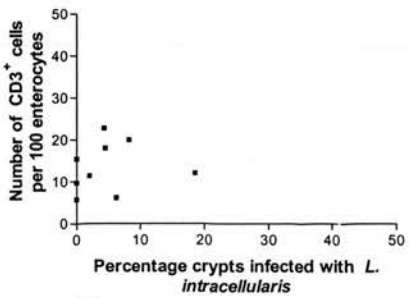

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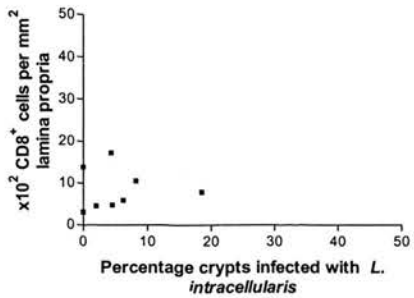

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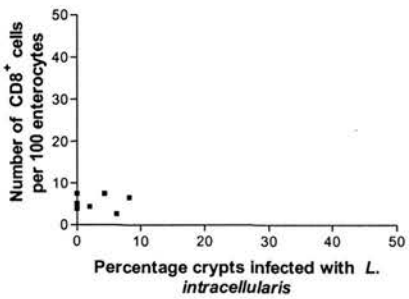

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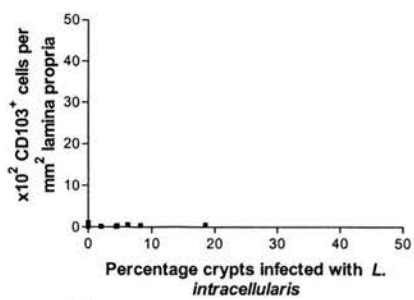

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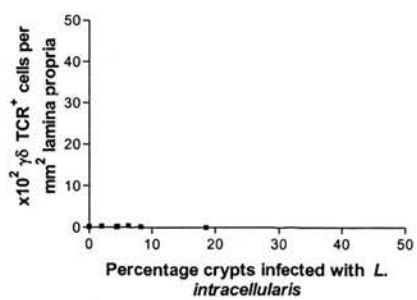

(f)

Appendix III. Correlation graphs following inoculation of WT mice with *L. intracellularis*. All analyses were performed on sections of colon and at 14 days post-inoculation because this was the only time point at which *L. intracellularis* was detected.
(a) $CD3^+$ cells per mm^2 lamina propria; (b) $CD3^+$ cells per 100 enterocytes; (c) $CD8^+$ cells per mm^2 lamina propria; (d) $CD3^+$ cells per 100 enterocytes; (e) $CD103^+$ cells per mm^2 lamina propria; (f) $\gamma\delta$ TCR^+ cells per mm^2 lamina propria.
Note the change in scale for the x-axis due to the lower number of crypts infected in WT mice.

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Gamma Interferon Influences Intestinal Epithelial Hyperplasia Caused by *Lawsonia intracellularis* Infection in Mice

DAVID G. E. SMITH,[1]* SUZANNE C. MITCHELL,[1,2] TONY NASH,[3] AND SUSAN RHIND[2]

Zoonotic & Animal Pathogens Research Laboratory, Department of Medical Microbiology,[1] Department of Veterinary Pathology, Easter Bush Veterinary Centre,[2] and Department of Veterinary Pathology, Summerhall,[3] University of Edinburgh, Edinburgh, Scotland

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***Lawsonia intracellularis* is a recently identified bacterial pathogen which causes disease in a broad range of animals. Invasion of intestinal epithelial cells and the resultant hyperplasia of infected cells are central processes in disease pathogenesis. In this study, we aimed to establish whether immunocompetent mice were susceptible to infection and whether gamma interferon (IFN-γ) contributed to the pathogenesis of infection. Wild-type 129-Sv-Ev mice (129 mice) and IFN-γ receptor knockout mice based on the 129 background (IFN-γR$^-$) were challenged orally with approximately 5.5×10^7 *L. intracellularis* cells. Both 129 and IFN-γR$^-$ mice became infected, although the extent of infection (as determined by the proportion of infected crypts) was substantially lower in 129 mice than in IFN-γR$^-$ mice. Despite these differences, infected crypts showed characteristics typical of proliferative enteropathies of other animals, i.e., intracellular colonization of epithelial cells by *L. intracellularis* with resultant epithelial hyperplasia. Infection in 129 mice was cleared between days 21 and 28 postchallenge, whereas infection in IFN-γR$^-$ mice was evident in 100% of animals from day 21 onward. Additionally, in IFN-γR$^-$ mice the infection was so extensive that fatalities resulted. IFN-γ therefore plays a significant role in limiting intracellular infection and increased cellular proliferation associated with *L. intracellularis*. *L. intracellularis* infection is generally associated with modest cellular infiltration; therefore, further comparative examinations will be necessary to determine pathogenicity factors and define the role of IFN-γ in controlling this infection.**

Lawsonia intracellularis is a recently identified intracellular pathogen which is phylogenetically unrelated to other pathogens (10, 26). It is an obligate intracellular enteropathogen and is the etiological agent of infectious intestinal hyperplasias for which several synonyms have been applied including proliferative enteropathy (PE), intestinal adenomatosis, and ileitis. *L. intracellularis* causes a disease complex which is primarily recognized in pigs (20, 36); however, as diagnostic methods improve, *L. intracellularis* is emerging as a cause of intestinal hyperplasia in an increasing range of mammalian and avian species (reviewed in reference 20). For example, *L. intracellularis* has most recently been reported as a cause of fatal enteritis in captive macaques (19). Previously it was also suggested to be a possible agent of ulcerative colitis in humans, although the etiological role was not established (31). On the basis of 16S rRNA gene sequences, *L. intracellularis* is most closely related to sulfate-reducing bacteria of the genus *Desulfovibrio* (7, 10, 30) and to *Bilophila wadsworthia* (20). Although the latter and some species of the former are intestinal inhabitants and occasional pathogens, there are no other apparent similarities to *L. intracellularis*.

L. intracellularis preferentially invades intestinal epithelial crypt cells, where the bacteria reside and replicate within the cytoplasm. Infection with most enteroinvasive bacteria commonly involves infiltration of inflammatory cells (polymorphonuclear leukocytes or monocytes), leading to foci of infection, epithelial necrosis and often dissemination to other sites (e.g., the liver). Although it also targets the intestinal epithelium, *L. intracellularis* is significantly different from the other enteroinvasive bacteria and exhibits unique pathological characteristics (20). The major recognized pathological consequence of infection is hyperplasia of infected crypts with negligible evidence of an inflammatory response (20). The organism is almost exclusively associated with the intestinal epithelium, exhibiting no detectable dissemination to other organs.

Intestinal epithelial hyperplasia is known to occur as a major pathological consequence of infection with only a small number of bacterial pathogens. In addition to *L. intracellularis*, these bacteria include *Helicobacter* species and *Citrobacter rodentium*, both of which are relatively well characterized in comparison to *L. intracellularis* (22, 27). Although the pathological characteristics of *L. intracellularis* infection are well documented (see e.g., references 20 and 36), it is apparent that this bacterium is a unique enteropathogen for which pathogenic mechanisms remain speculative. Currently we have only rudimentary knowledge of either the host or bacterial factors which contribute to proliferative enteropathies caused by *L. intracellularis*.

Gamma interferon (IFN-γ) is central to the progression of infection by intracellular bacteria and other intracellular pathogens, as demonstrated by a variety of approaches. For instance, transgenic mice with specific deletions in IFN-γ or IFN-γ receptor (knockouts) or treated with antibodies specific for IFN-γ demonstrate increased susceptibility to challenge with, for example, *Salmonella enterica* serovar Typhimurium (11, 29), *Shigella* spp. (47), or *Listeria monocytogenes* (3). Since *Lawsonia* infection is relatively noninflammatory and induces increased epithelial proliferation, the cytokine regulatory networks and cascades are likely to differ from those induced by invasive inflammatory bacteria. Similarly, the roles of host cellular defenses are likely to differ.

In order to examine the possible role of IFN-γ in *Lawsonia*

* Corresponding author. Mailing address: Zoonotic & Animal Pathogens Research Laboratory, Department of Medical Microbiology, University of Edinburgh, Scotland, EH8 9AG, United Kingdom. Phone: 44 131 651 1343. Fax: 44 131 650 6531. E-mail: dgesmith@vet.ed.ac.uk.

infection, we established an infection system in mice using bacteria cultured in vitro and examined infection and hyperplasia in IFN-γ receptor-deficient and isogenic wild-type mice.

MATERIALS AND METHODS

Bacteria and preparation of inoculum. *L. intracellularis* isolate LR189/5/83 was originally obtained from a pig with porcine proliferative enteropathy in the United Kingdom (21). Bacteria were isolated from homogenized intestinal tissue and grown in cell culture in IEC-18 intestinal epithelial cells by methods described previously (21). Following isolation and serial passage in the laboratory for up to nine passages, this strain can reproduce disease in experimentally inoculated pigs (42, 43). From pure, frozen stocks of bacteria, *L. intracellularis* was cultured to a final number of 11 passages; cells were then lysed and bacterial suspensions were prepared in SPG (sucrose-potassium-glutamate) buffer with 5% fetal calf serum. To enumerate *L. intracellularis*, infected-cell lysate was serially diluted for infection of IEC-18 cells on 13-mm coverslips (carried out in triplicate). Numbers of infectious units (IU; equating to viable bacteria) in this suspension were estimated as a product of the number of infected cells, dilution, and an estimated number of 50 bacteria per infected cell (based on previous observations). Detection of *L. intracellularis* in infected monolayers was carried out by our routine immunodetection methods (21) using a monoclonal antibody specific for an *L. intracellularis* surface antigen (27). The purity of bacterial suspensions (including freedom from mycoplasmas and *Chlamydia*) was confirmed as described previously (21). Aliquots of suspension containing approximately 2.2×10^8 *L. intracellularis* IU · ml^{-1} were stored at −70°C and thawed immediately prior to inoculation of animals.

Mice and experimental design. All procedures were conducted in accordance with the guidelines of the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 and approved by an Ethical Review Committee. Mouse strains challenged were of the 129-Sv-Ev genotype (wild type, designated 129) and IFN-γ receptor knockout mice based on this background (IFN-γR$^-$) (16). Cohorts of mice were regularly screened for pathogens of laboratory rodents and were maintained under specific-pathogen-free conditions. At the time of challenge the animals were of mixed genders and aged 47 to 62 days old. The mice were dosed with 0.25 ml of *L. intracellularis* suspension (four mice per challenge group, with each mouse receiving approximately 5.5×10^7 IU) or with 0.25 ml of SPG buffer (control group) by oral gavage with a ball-ended 21-gauge needle. The animals were given access to water and feed ad libitum and were monitored daily for health status. At each required time point postinfection (14, 21, 28, and 35 days), groups of four mice inoculated with *L. intracellularis* and one control mouse were removed for examination. Following euthanasia by CO_2 asphyxiation, the animals were weighed and a postmortem examination was carried out as described below.

Monitoring of infection and pathological examinations. Multiple regions of small and large intestine were removed at postmortem, sectioned longitudinally, and examined for evidence of intestinal thickening or hemorrhage. Samples were either fixed in 10% neutral buffered formalin or mounted in OCT compound (Merck) and snap-frozen in a slurry of dry ice and isopentane. The formalin-fixed tissue was trimmed, dehydrated through graded alcohol concentrations, and embedded in paraffin wax, and 4-μm-thick sections were cut. These were stained with hematoxylin and eosin for routine histopathological examination. For immunohistochemistry, 4-μm sections were cut onto treated slides (BioBond; Biocell) and rehydrated through graded alcohol concentrations, and labeling was carried out using an indirect immunoperoxidase technique. Samples comprising mesenteric lymph nodes, spleen, and liver were also taken, mounted, and snap-frozen as above. Frozen material was cut as 6-μm sections on a cryostat, mounted, and air dried for 1 to 2 h at room temperature. Slides were fixed in acetone for 10 min, air dried twice, and then used immediately for immunohistochemical detection of *Lawsonia*.

Rabbit polyclonal serum 1080/76 (raised against a field isolate of *L. intracellularis*) was used as primary antibody, and detection was performed using anti-rabbit immunoglobulin-peroxidase conjugate (Vectastain Elite ABC kit; Vector Labs) as specified by the manufacturer. Following this, sections were counterstained with haematoxylin and then mounted in DePex (Merck). Tissues were examined histologically for features typical of *L. intracellularis* infection, i.e., intestinal epithelial hyperplasia and intracellular *L. intracellularis*. The relative proportion of infected crypts in the ileum and colon was determined to establish the extent of infection; the proportion of infected (and hence hyperplastic) crypts was counted over 10 random fields under a magnification of ×250.

RESULTS

Clinical and pathological determinations. Weight loss or failure to gain weight is characteristic of natural PE; however, this is a variable feature of experimental challenge of pigs with *L. intracellularis* (28, 42). This was borne out in our own experimental challenges of mice, where one of the two series had far greater clinical impact on mice than the other did although

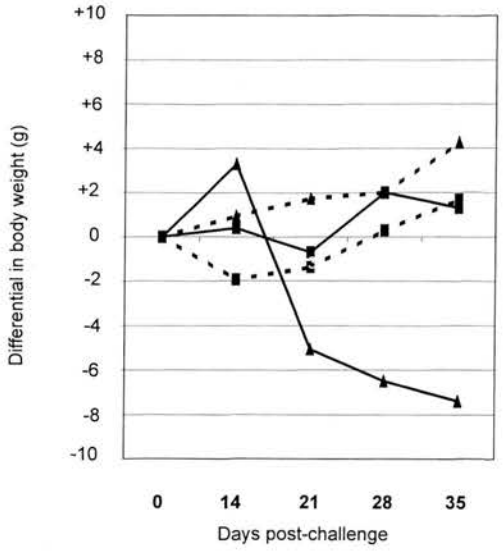

FIG. 1. Weight differentials in 129 (■) and IFN-γR$^-$ (▲) mice following oral inoculation with 5.5×10^7 IU of *L. intracellularis* (——) or carrier buffer (– – –). Zero on the *y* axis represents the mean weight of mice at inoculation. Values represent the means obtained from four mice at each time point except on day 35 in the IFN-γR$^-$ mice, when only three mice were weighed; see the text for details. Figures on the *x* axis represent days postchallenge.

the proportions of infected mice were similar. The differences cannot be ascribed to differences in the ages or genders of the mice or in the *L. intracellularis* inocula, which were similar in each series. The reasons for the variations in disease in these replicates, whether in mice, pigs, or hamsters, are unclear. Over the course of these challenges, the weights of infected 129 mice were lower than those of the control (sham-inoculated) mice on days 14, 21, and 28 postchallenge; however, these differences represented less than 10% of mouse body weight and were not significant. In IFN-γR$^-$ mice there were substantial differences in weights, and the results of one of two challenge experiments are presented in Fig. 1. The mean weights of challenged IFN-γR$^-$ mice were significantly lower after day 14 postchallenge, with the reduction in the average weight for the group (compared to uninoculated control) representing 29, 35, and 44% of body weight on days 21, 28, and 35, respectively.

At some time points, gross pathological examination of mice demonstrated significantly enlarged intestines (a feature typical of PE in other animals) in a at least one of the mice per group. In the affected animals, intestinal enlargement was evident in both ileum and colon, with the latter being more profoundly enlarged. Figure 2 shows typical examples of enlarged intestines in both wild-type 129 and IFN-γR$^-$ mice. Mucosal hemorrhage in the intestine is an occasional feature of some components of the PE disease complex (hemorrhagic enteropathy), and this was observed in the intestine from one of the IFN-γR$^-$ mice (not shown).

Characteristics of *L. intracellularis* infection in mice. Specific detection of *L. intracellularis* was carried out on intestinal sections by immunohistochemistry, the accepted standard for confirmation of infection by this bacterium. Characteristic intracellular bacteria and associated epithelial hyperplasia were detected in ileum and colon specimens in all mice exhibiting intestinal enlargement (Fig. 3). *L. intracellularis* was detected in both wild-type 129 and IFN-γR$^-$ mice, although the extent of infection was greater in IFN-γR$^-$ mice. *Lawsonia* was not

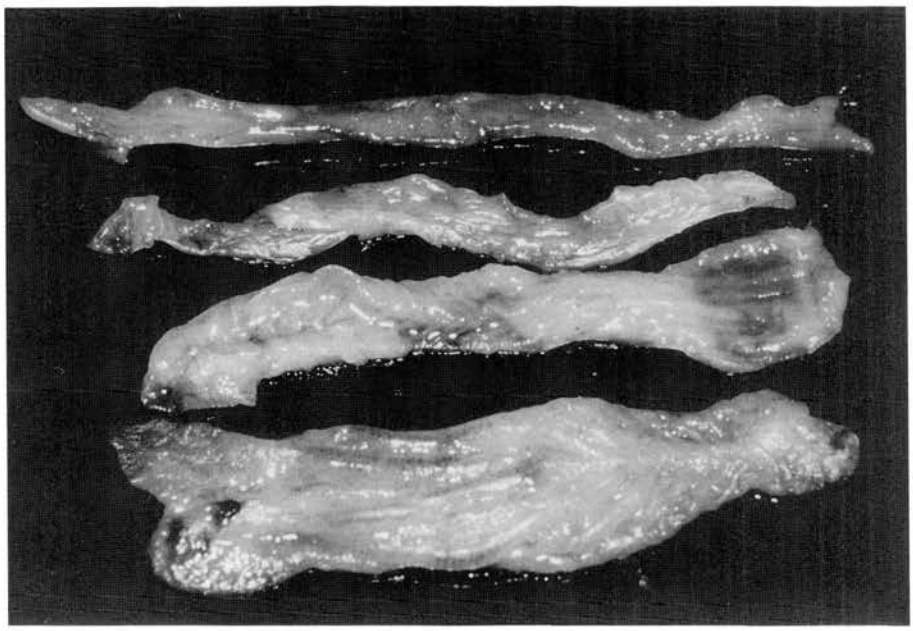

FIG. 2. Mouse colons obtained on day 28 postchallenge, showing characteristics typical of *L. intracellularis* infection. From top to bottom: 129 mouse sham challenged; IFN-γR$^-$ mouse sham challenged; 129 mouse challenged with *L. intracellularis*; IFN-γR$^-$ mouse challenged with *L. intracellularis*.

detected in the mesenteric lymph nodes, livers, or spleens of any of the mice examined (including IFN-γR$^-$ mice which died as a result of extensive *Lawsonia* infection in the intestine [see below]).

Immunohistological detection was used to determine the proportion of mice infected with *L. intracellularis* (strain designation LR189/5/83) at each time point postchallenge (Fig. 4). Both wild-type and IFN-γR$^-$ mice became infected and exhibited intracellular bacteria and hyperplastic crypts. In wild-type mice, infection was detected from the first sample time point (day 14) and on day 21 postchallenge. After this point, these intracellular bacteria were not detectable by immunohistochemistry. Similar results (not shown) were obtained in previous experimental challenges of 129 and IFN-γR$^-$ mice. In these preceding experimental challenges, bacteria were not observed in either the colon or ileum on day 7 postinfection; therefore, this and ethical considerations led to omission of this time point from this series of experiments. By comparison, the C57BL/6 strain of mice (results not shown) exhibited similar susceptibility to infection to that of 129 wild-type mice. The observed pattern of infection in mice mimicked that observed in experimentally infected pigs (18, 24, 42, 43), where intracellular infection becomes demonstrable on days 10 to 14 postchallenge and resolves 2 weeks or more later.

The extent of infection in the ileum and colon was determined by counting the proportion of infected (and hence hyperplastic) crypts over 10 random fields under a magnification of ×250. In wild-type 129 mice, levels of infection in the ileum and colon were low, showing, respectively, 1.3 and 3.5% of crypts infected on day 14 postchallenge. IFN-γR$^-$ mice showed a higher susceptibility to infection and a progressive increase in infected crypts in both ileum and colon to day 28 postchallenge. The proportion of infected colonic crypts on days 14, 21, 28, and 35 were 10, 60, 69, and 40%, whereas the corresponding values in the ileum were 10, 5, 44, and 0%, respectively. The proportion of infected crypts reached 100% in mice which died as a result of challenge. An example of this extensive infection of IFN-γR$^-$ mice is shown in Fig. 2.

Three of the four IFN-γR$^-$ mice from the group for the final time point (scheduled for day 35) died or were euthanized in extremis between days 31 and 32 postchallenge, representing a fatality rate of 75%. These animals were excessively anorexic, showing a reduction in body weight to approximately 50% of that of controls. Immunohistological examination of the colon and ileum from these mice indicated extensive epithelial infection with *L. intracellularis*, with nearly 100% of epithelial cells affected in 100% of crypts. Even in the mice that died, there was no evidence of bacterial dissemination to the liver or spleen.

DISCUSSION

In this study we have demonstrated unequivocally that immunocompetent, wild-type 129-Sv-Ev mice are susceptible to infection with *L. intracellularis* under experimental conditions although infection was detectable only at a low level, as indicated by the proportion of infected crypts. Previously it has been shown that pigs (18, 24, 42, 43) and hamsters (17, 44) are both susceptible under experimental conditions but that mice, rats, and chickens are highly refractory (1). In the present study, not only were inoculated mice successfully colonized by *L. intracellularis* but also infection led to clinical and pathological features characteristic of PE in other animals, i.e., intracellular bacteria, epithelial hyperplasia, and (in some mice) anorexia. This extends further the range of animals susceptible to enteric disease caused by this bacterium. The range of animals in which natural infection and associated disease has been detected has broadened over the last decade and now includes pigs, hamsters, ferrets, foxes, deer, rabbits, horses, emus, ostriches, macaques, and others (reviewed in reference 20). Although to our knowledge natural infection has not been detected in either wild or laboratory mice, the former represent a potential reservoir; hence, our findings have further implications for the ecology and epidemiology of PE, particularly in pigs, where the environment is likely to be cohabited by rodents.

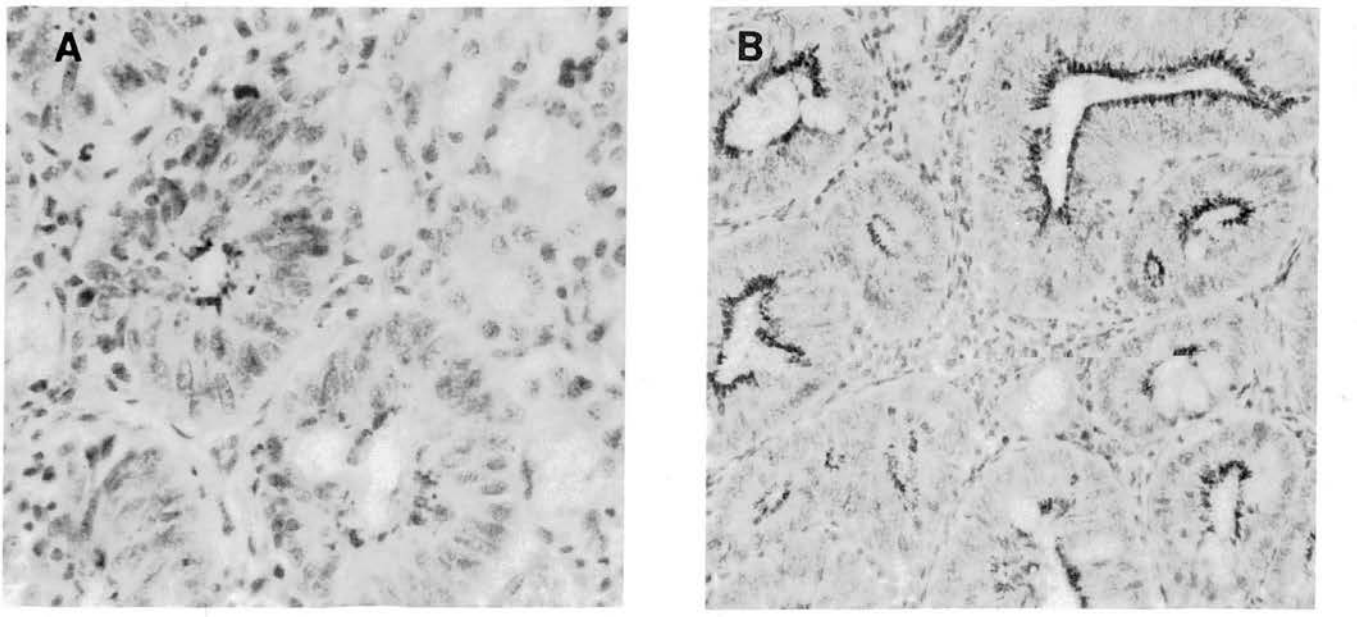

FIG. 3. Immunochemistry of colonic tissue for *L. intracellularis* antigen, counterstained with hematoxylin. Dark-staining areas represent *L. intracellularis* organisms within epithelial cells. Infected crypts show epithelial hyperplasia. Original magnification, ×250. (A) Wild-type 129 mouse on day 28 post-challenge; (B) IFN-γR$^-$ mouse on day 28 postchallenge; C, IFN γR$^-$ mouse that died (day 32 postchallenge).

Although pigs have been considered the major susceptible animal, it is becoming increasingly apparent that *L. intracellularis* has a broad host range which is potentially wider than currently known. Despite evidence of infection in primates, there is currently no direct evidence that *L. intracellularis* can infect humans, although one report (31) has suggested a link with human disease. In view of the known broad host range of *L. intracellularis* and the wide spectrum in clinical presentation (20, 36), we should remain alert to the possibility that the range of susceptible species may broaden further and may incorporate humans.

In the mice examined, the principal site of infection, as demonstrated by gross pathology, histology, and immunohistochemistry, was the colon rather than the ileum. The main site of infection (and hence hyperplasia) differs among the animals in which natural disease has been reported (2, 4–6, 8, 15, 20, 23, 30, 41, 48). This may reflect physiological or immunological differences between the animals. Additional factors may be the differing commensal populations or expression of particular bacterial receptors in those sites. Which, if any, of these possibilities are involved remains to be determined; however, it has been shown that commensal populations are required for successful infection with *L. intracellularis* (25), a factor which does not exclude any of the above possibilities. In none of the mice (even those which died or were euthanized in extremis) was *Lawsonia* detectable in sites other than the intestinal epithelium. This confirms the absolute specificity of the tropism of *L. intracellularis* for intestinal epithelial cells, particularly immature (crypt) cells, even in animals in which Th1 cell-mediated responses are diminished.

The general pattern of infection in wild-type mice resembled that seen in experimentally infected pigs in previous studies (18, 24, 42, 43). Epithelial infection becomes detectable on around day 14 postchallenge, remains detectable for a variable period thereafter, and then resolves spontaneously. The presence of detectable intraepithelial *L. intracellularis* coincides with the development of a primary serological response to major surface antigens (S. C. Mitchell, N. MacIntyre, J. R. Thomson, S. Rhind, and D. G. E. Smith, unpublished data). Resolution of infection presumably coincides with developing immune (or other defense or repair) responses; however, further detailed examinations of immune responses are necessary to corroborate this.

Histologically, disease in experimentally challenged mice closely corresponds to disease in other animals, showing intracellular bacteria and epithelial hyperplasia in infected crypts. These studies also suggest that infection is accompanied by some cellular infiltration in mice, at least in the 129-Sv-Ev genetic background (results not shown). Although it is gener-

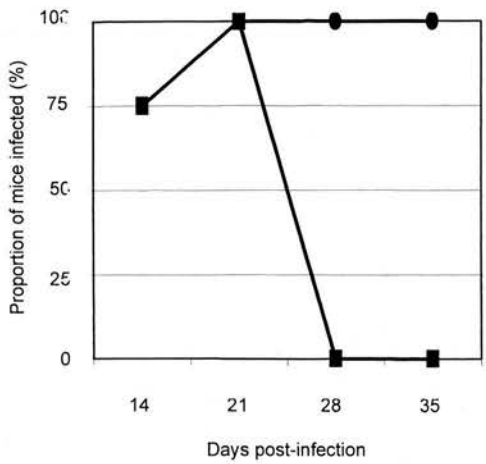

FIG. 4. Proportions of wild-type 129 (■) and IFN-γR$^-$ (●) mice infected over the duration of the challenge study as determined by immunohistochemical examination of ileal and colonic mucosa.

ally accepted that uncomplicated proliferative enteropathy in pigs is not accompanied by extensive leukocyte infiltration, such responses have been observed in other animals.

It is possible that other strains of mice may respond in a different manner from that of 129-Sv-Ev mice; however, the contribution of mouse genotype (other than IFN-γ receptor) to immune responses and susceptibility was not an objective of this study since they are governed by multiple genetic factors. Preliminary data, however, do show that wild-type mice of the 129 and C57BL/6 (results not shown) backgrounds are both colonized intracellularly by *L. intracellularis* with resultant hyperplasia. Our results with C57BL/6 mice conflict with a previous report (1) which failed to demonstrate any susceptibility of these mice to infection with this bacterium. These differences in apparent susceptibility between these two studies cannot be ascribed to differences in inocula, which were numerically similar. A study of the influence of inoculum, bacterial strain, and mouse genotype on susceptibility and a detailed examination of immune responses have yet to be conducted.

IFN-γ, as well as other effectors, is elicited in the early phase in pathogenesis of other enteroinvasive bacteria (3, 11, 29, 32, 47) and is important in both the progression and control of epithelial infection. Given the important role of IFN-γ in other intracellular bacterial infections, we sought to establish whether IFN-γ played a role in the control of this intracellular infection. Mice in which IFN-γ receptor was deleted (IFN-γR$^-$) demonstrated a markedly increased susceptibility to experimental *L. intracellularis* challenge. Rates of infection were higher in IFN-γR$^-$ mice than in wild-type mice: whereas in wild-type mice there was no evidence of infection by day 28 postchallenge, all IFN-γR$^-$ mice in each group demonstrated infection for the 35-day duration of the study. IFN-γR$^-$ mice also showed a far greater proportion of infected crypts (and consequently hyperplasia) compared to wild-type mice. Additionally, fatalities resulting from *Lawsonia* infection occurred around day 31 postchallenge in 75% of IFN-γR$^-$ mice in one challenge series. Despite the differences in pathological presentation between *L. intracellularis* and other enteroinvasive bacteria, it thus appears that IFN-γ is important in the control of *L. intracellularis* infection as well as of infections caused by other intracellular pathogens. IFN-γ has multiple possible roles in limiting and controlling intracellular infections and may contribute to the control of *Lawsonia* infection by exerting effects directly upon epithelial cells, by immune-mediated mechanisms, or by a combination of means. The possible roles for IFN-γ in control of *L. intracellularis* infection remain to be pursued.

From the current investigation, it was evident that there was a marked increase in crypt hyperplasia in *L. intracellularis*-infected IFN-γR$^-$ mice compared to wild-type mice. The absence of a functional IFN-γ receptor in the IFN-γR$^-$ mice therefore led to enhanced epithelial proliferation during *L. intracellularis* infection. The involvement of IFN-γ in regulating epithelial cell differentiation and proliferation has been noted previously (37, 46). Despite the demonstration of the involvement of IFN-γ in suppression of proliferation, its role in regulating hyperplasia caused by bacteria is complex. In an investigation of murine colonic hyperplasia (13), infection of IFN-γR$^-$ mice with *Citrobacter rodentium* failed to produce hyperplasia. Additionally, in the IFN-γR$^-$ mouse background, massive lymphocyte infiltration, which is typical of infection in the wild-type background, did not occur. IFN-γ and lymphocyte infiltration are also involved in *Helicobacter* infection (12, 35, 38, 45). Infection with *L. intracellularis*, in contrast, is not noted to correspond to such extensive inflammatory infiltration, and in IFN-γR$^-$ mice infection resulted in amplification of hyperplasia rather than abrogation. This indicates that there is an apparent dichotomy in the potential roles of IFN-γ in hyperplasia induced by these bacterial infections. We are continuing to examine in detail the lymphocyte responses in situ in both mice and pigs infected with *L. intracellularis* and their contribution to control and pathology.

Characterization of *Lawsonia*-induced hyperplasia will provide interesting insights since differences in the apparent roles of IFN-γ and lymphocytes may be reflected in other mechanistic distinctions. For instance, increased epithelial cell proliferation in infections caused by *Helicobacter* spp. and *C. rodentium* is associated with epidermal growth factor-like (33, 34) factors and keratinocyte growth factor (13, 14), respectively. We have yet to determine whether these or other similar growth factors are involved in *Lawsonia* infection. Similarly, direct involvement of bacterial effectors has been demonstrated to induce hyperplasia by both *Helicobacter* spp. (45) and *C. rodentium* (13, 14). Investigations of *L. intracellularis* to date have not detected sequences homologous to intimin (D. G. E. Smith, B. W. Wren, and J. Hannigan, unpublished data), which is essential for pathological changes associated with murine colonic hyperplasia (9, 39, 40), although we have yet to examine this bacterium for determinants homologous to those conferred on the *Helicobacter* pathogenicity island. It appears, therefore, that although there is functional convergence there is no paradigm for bacterially induced hyperplasia since the mechanisms underlying *L. intracellularis*-induced epithelial hyperplasia differ from those in *Helicobacter* and *C. rodentium* infection. The bacterial and host factors involved in *L. intracellularis* infection remain to be ascertained; however, further comparative examinations of bacterial and host factors involved will be beneficial to the advancement of understanding and control of growth disturbances.

In this study we have established that mice are susceptible to infection with *L. intracellularis*, at least under the experimental conditions employed. We have thus developed an in vivo model in which some of the characteristics of intestinal hyperplasia caused by this bacterium can be further analyzed. Through application of this experimental infection system, we have initiated a definition of host factors which contribute to both the pathology and control of disease caused by *L. intracellularis*. This bacterium is one of a range of diverse prokary-

otic enteropathogens which have evolved life-styles which involve invasion of the intestinal epithelium; however, it is unique among these in inducing epithelial hyperplasia as its major pathological consequence without substantial leukocyte involvement. Induction of epithelial hyperplasia appears to be essential to *L. intracellularis* since bacterial proliferation and host cell replication are interdependent (22), a feature which represents an unusual evolutionary adaptation significantly different from that of other enteroinvasive bacterial pathogens.

This investigation is the first to establish categorically that mice are susceptible to infection with this bacterium and that, as in other intracellular bacterial infections, IFN-γ is crucial in the control of *L. intracellularis* infection, although the mechanism by which this control is exerted has yet to be defined. This bacterium is also unique among the small group of enteropathogenic bacteria which induce epithelial hyperplasia, and our investigations also make it apparent that no single mechanism is responsible for this kind of pathological alteration. It is clear that host mechanisms in these infections are complex and require further elucidation. Since there are apparently a multiplicity of routes by which disturbances in cellular growth and differentiation may arise, *L. intracellularis* represents another organism useful in the examinations of epithelial hyperplasia and its control. Our establishment of infection in the mouse offers a valuable system to continue investigations of immune and other regulatory mechanisms in this and other intestinal hyperplastic conditions.

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